ATTEMPTS to ENGINEER PARTHENOGENESIS in *Arabidopsis thaliana*

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. Nat)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Miłosława Jaciubek

aus

Grudziądz, Polen

Promotionskomitee

Prof. Dr. Ueli Grossniklaus

Prof. Dr Beat Keller

Prof. Dr. Charles Spillane

(Centre for Chromosome Biology, NUI Galway, Ireland)

Zürich, 2012

ZUSAMMENFASSUNG

Bedecktsamer (Angiosperme) reproduzieren sich sexuell und ihr Lebenszyklus alterniert zwischen einem diploiden, dominanten Sporophyt und einem haploiden, reduzierten Gametophyt. Die Aufgabe des Sporophyten ist die Ausbildung zweier haploider Sporen welche sich in weibliche und männliche Gametophyten weiterentwickeln. Der weibliche Gametophyt (Embryosack) besteht typischerweise aus zwei Gametenzellen (Ei- und Zentralzelle) und weiteren fünf Zellen (Zwei Synergiden- und drei Antipodenzellen). Der männliche Gametophyt (Pollen) besteht aus zwei Spermzellen und einer vegetativen Zelle. Vor der Befruchtung bildet diese vegetative Zelle einen Pollenschlauch aus, um die darin eingeschlossenen Spermzellen vom Stigma bis zum weiblichen Gametophyten zu transportieren. Während der doppelten Befruchtung fusioniert eine der Spermzellen mit der Eizelle zur diploiden Zygote, und die andere Spermzelle mit der diploiden Zentralzelle zu einer triploiden Endospermzelle. Aus der Zygote entsteht in der Folge der Embryo und aus der Endospermzelle das Nährgewebe des sich entwickelnden Samens.

Einige Angiosperme habe auch alternative Mechanismen zur Fortpflanzung durch Samen entwickelt. Apomixis ist eine asexuelle Reproduktion durch Samen, die nächste Generation wird ohne männlichen Beitrag zum Genom gebildet. Die entstehenden Nachkommen sind natürliche Klone. Apomixis hat schon lange das Interesse von Pflanzenzüchtern auf sich gezogen, weil dieser Fortpflanzungsmechanismus in Kulturpflanzen die Erzeugung und Erhaltung von komplexen Hybriden erheblich vereinfachen könnte. Die Vielfalt der beschriebenen apomiktischen Prozesse zeigt, dass Apomixis mehrmals unabhängig in der Evolution entstanden ist. Aus entwicklungsbiologischer Sicht kann Apomixis als zeitlich und räumlich deregulierter sexueller Prozess betrachtet werden: die Megasporen entwickeln sich ohne meiotische Reduktionsteilung (Apomeiosis), die Eizelle bildet ohne Befruchtung einen Embryo und ein funktionelles Endosperm entwickelt sich.

Die genetischen Komponenten der Apomixis sind weitgehend unbekannt. Weder Mapping-Ansätze noch das Einkreuzen dieses Merkmals von wilden apomiktischen Verwandten in Kulturpflanzen waren darin bisher sehr erfolgreich. Die Forschung mit sexuellen Model-Pflanzen wie Arabidopsis oder Mais ermöglicht Einblicke in diese

Form der Reproduktion. Mehrere Mutanten welche Aspekte apomiktischer Prozesse aufweisen wurden beschrieben.

In dieser Arbeit wurden die molekularbiologischen Möglichkeiten in *Arabidopsis thaliana* eingesetzt in dem Versuch durch De-regulation der Genexpression in der Eizelle erste Schritte einer parthenogenetischen Fortpflanzung auszulösen. Dabei wurden mehrere Ansätze parallel verfolgt.

Ein Gain-of-function Screen wurde in einer männlich sterilen Pflanzenlinie durchgeführt. Mit einem Zwei-Komponenten System wurden spezifisch im Eiapparat zufällig markierte Genen überexprimiert. Der chimäre Transkriptionsfaktor XVE wird zell-spezifisch exprimiert. Im Eiapparat dimerisiert XVE nach der Induktion mit humanem Östrogen, transloziert in den Zellkern und bindet an die künstliche Zielsequenz des LexA-Operators um die Transkription benachbarter Gene zu initiieren. Aus über 9000 analysierten individuellen Transformanden wurde kein reproduzierbarer parthenogenetischer Phänotyp identifiziert.

In einem weitern Ansatz wurden acht Faktoren der Zellzyklusprogression im Eiapparat dereguliert. Während der G1/S Progression führt die Suppression des RETINOBLASTOMA RELATED PROTEIN1 (RBR1) zur Freisetzung dieser Faktoren, welche für die Zellzyklusprogression wichtig sind. In der lichtmikroskopischen Analyse der induzierten Linien wurden jedoch keine auf Parthenogenese hindeutenden Phänotypen identifiziert.

Nebst der indizierbaren Expression von Genen wurden erste Schritte unternommen, das Zwei-Komponenten System für induzierbares Gen-Silencing mit Hilfe von amiRNAs zu etablieren. Für RBR1 und MULTICOPY SUPPRESSOR OF IRA1 (MSI1) wurden induzierbare amiRNAs hergestellt. MSI1 schient vor der Befruchtung zu verhindern, dass sich die Eizelle in eine Zygote entwickelt, spielt danach aber auch in der Embryoentwicklung eine Rolle. Ein Knock-down in der unbefruchteten Eizelle könnte demnach eine frühzeitige Ausbildung eines Embryos auslösen. Das indizuerbare amiRNA System befindet sich noch in der frühen Entwicklungsphase und benötigt noch weitere Experimente und Kontrollen, um fundierte Aussagen treffen zu können.

SUMMARY

Angiosperms reproduce sexually and their life cycle alternates between a dominant diploid sporophyte and a reduced haploid gametophyte. The major role of the sporophyte is a production of haploid spores that develop into the female and male gametophytes, respectively. The female gametophyte (embryo sac) typically consists of gametes (the egg and the central cell) and accessory cells (two synergds and three antipodal cells). The male gametophyte (pollen) consists of two sperm cells and a vegetative cell. Upon fertilization, the vegetative cell generates a pollen tube to deliver two sperm cells to the female gametophyte. During the process of double fertilization one sperm cell fuses with the egg cell giving rise to the embryo, whereas the other fertilize the central cell resulting in the development of the nourishing tissue, the endosperm.

Angiosperms developed also an alternative mechanism of propagation defined as apomixis. It is an asexual reproduction through seeds where the next generation is produced without a paternal contribution. The resulting offspring is genetically identical with the maternal component and represents a natural type of the clonal propagation. Apomixis receives a great interest from plant breeders. Installing of apomixis in important agricultural crops could facilitate the generation and propagation of highly complex hybrids. The genetic mechanisms that govern apomixis are still unknown. The diversity of reproductive mechanisms found in apomictic species suggests that apomixis evolved many times independently during evolution. From a developmental point of view, apomixis is thought to be a deregulation of sexual processes in time and space. In apomictic plants the endosperm develops either autonomously or its development requires fertilization of the central cell. Mapping approaches to identify apomictic genes revealed large non-recombining regions hampering this approach. Attempts to introgress apomixis from wild relatives into crops have been unsuccessful. In addition to studies in natural apomictic species, sexual model plants can provide insight into the apomixis trait. Several loss-of-function mutants were identified in Arabidopsis and maize that display elements of apomictic development. However, the fully apomictic plant was not obtained.

Here, using the powerful molecular tool of the sexual model plant *Arabidopsis thaliana* we attempted to engineer parthenogenesis by the deregulation of the gene

expression in the egg apparatus. An egg apparatus specific gain-of-function screen in the male sterile background was initiated to identify genes that may be required for the parthenogenetic embryo development. An inducible two component system was used. This system relies on the inducible chimeric transcription factor XVE and its O*lexA* binding domain. An introduction of an egg apparatus specific enhancer (*AtEASE*) restricts the XVE expression to the egg apparatus. Upon induction with human estrogen XVE binds to the *lexA* domain and induces expression of genes adjacent. Screening of ~9 000 mutants did not results in isolation of a mutant that displays the parthenogenetic embryo development in absence of fertilization.

In addition, a candidate gene approach was used in order to engineer parthenogenesis in *Arabidopsis thaliana*. We verified genes of the G1/S phase of the plant cell cycle as potential candidates that can trigger the parthenogenetic embryo development in the Arabidopsis embryo sac. During the G1/S transition the suppression of the RETINOBLASTOMA RELATED PROTEIN 1 (RBR1) results in releasing of the transcription factors that promote the cell cycle progression. We overexpressed eight genes that positively regulate the G1/S phase in the egg apparatus of the male sterile mutant in order to induce parthenogenesis. Microscopic observations of the female gametophyte did not reveal developing embryos. Attempts to silence the *RBR1* gene product in the egg apparatus by artificial microRNA failed. We did not obtain mutant plants.

Artificial micro RNA method was used to silence the *MULTICOPY SUPPRSSOR OF IRA 1* (*MSI1*) gene in the Arabidopsis egg apparatus. Before fertilization the MSI1 gene may be involved in the suppression of the egg cell activation, while after fertilization is required for the embryo development. We hypothesized that silencing of the *MSI1* in the egg apparatus may result in parthenogenesis. Microscopic observations of the embryo sac did not revealed parthenogenetic embryos in obtained mutant lines. However, this experimental approach is still in the process to be established and controls are required to in order to make scientific statements.

CURRICULUM VITAE

Persönliche Daten

Name	JACIUBEK
Vorname	Miłosława
Geburtsdatum	29.03.1976
Nationalität	Polnisch

Schulbildung

Mai 1995	König-Jan-III-Sobieski-Allgemeinbildenden Lyzeum Nr. II in Grudziądz, Polen in der Klasse mit humanistischem Profil
Okt 1998 - Juli 2003	Studium in der Fachrichtung Landwirtschaft an der Landwirtschaftlichen Universität Szczecin, Polen Diplomarbeit: "Linkage analysis of RAPD markers and genes controlling male sterility in Bc1 progeny of interline hybrid of rye with the CMS-C cytoplasm" Am Departement für Genetic und Pflanzenzüchtung Betreuer: Dr. ing. Stefan Stojałowski
11. Juli 2003	Abschluss als Magister Ingenieur
Feb. 2007 - heute	Eingeschrieben als Doktorandin an der Universität Zürich

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Angiosperm Life Cycle

The flowering plants (Angiosperms) reproduce sexually and their life cycle alternates between the dominating diploid sporophytic and the haploid gametophytic generation that differ morphologically and functionally (Gifford and Foster, 1989). As a result of the adaptation to life on land, in higher plants the sporophyte became the dominant phase, whereas the gametophytic generation producing the gametes became reduced to the very small size and develops within the sexual organs of the flower (Figure 1-1).

Figure 1-1. *Lilium* L. flower morphology. The typical flower consists of four whorls of organs: sepals, petals, stamens and pistils. The very outer whorl in of the lily flower consists of tepals, which includes sepals and petals. The male reproductive organ the stamen consists of a long stalk, the filament, with the anther at the tip. The anther is a structure where pollen grains are produced. The female reproductive organ, the pistil is typically located in the center of the flower. It consists of a swollen base, the ovary containing ovules, the style arising from the ovary and a pollen- receptive tip, the stigma.

The major role of the sporophyte generation is a production of two types of the haploid spores that subsequently develop into the female and male gametophytes, respectively. The female gametophyte, or the embryo sac, develops within the ovule located in the carpel's ovary. The male gametophyte or the pollen grain is formed in the stamen's anther. In the gametophytes the production of the haploid gametes for fertilization takes place. The fertilization process brings together the male and female

gametes which results in a zygote formation that is the next diploid sporophytic generation, thus completing the life cycle (Gifford and Foster, 1989; reviewed in Drews and Yadegari, 2004).

1.1.1. Female Gametophyte Development

Among the angiosperms, more than 15 different patterns of the female gametophyte development were described (Maheshwari, 1950; Huang and Russell, 1992). Major differences between them result from the variations in cytokinesis during meiosis, the number of mitotic division and the pattern of celullarization during gametogenesis (Yadegari and Drews, 2004). The most common structural and developmental pattern of the female gametophyte in angiosperms is the *Polygonum* type, first described in *Polygonum divaricatum*. This pattern of the gametogenesis is found in more than 70 % of flowering plants, including many important crop species like wheat or potato as well as in the model plant *Arabidopsis thaliana* (Maheshwari, 1950; Huang and Russell, 1992).

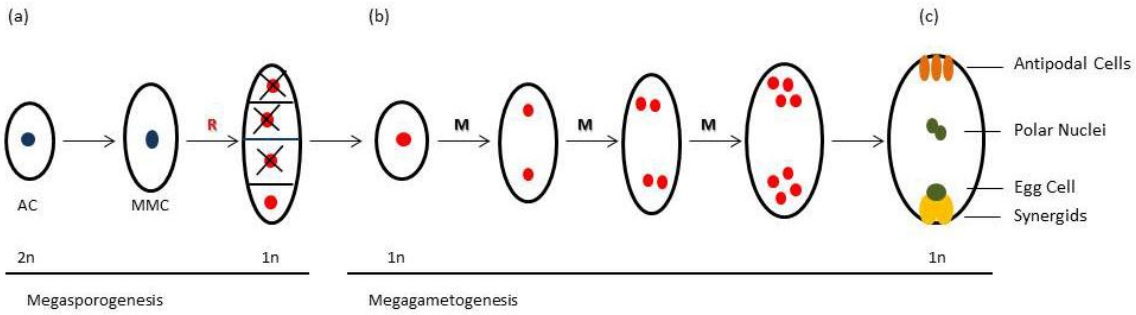


Figure 1-2. The *Polygonum*-type female gametophyte development. (a) During megasprogenesis, archesporial cell (AC) gives rise to the megaspore mother cell (MMC), which subsequently undergoes meiotic divisions leading to tetrad of megaspores. Only one megaspore survives to form a functional megaspore. (b) During megagemetogenesis, functional megaspore divides mitotically to form multicellular haploid embryo sac. (c) The mature embryo sac of the *Polygonum* type consists of seven cells: two synergides, one egg cell, one central cell and three antipodal cells. The nuclei in red are reduced (haploid) whereas in blue unreduced (diploid).

Regardless of the developmental pattern, the female gametophyte development in Angiosperms can be divided into the two main developmental phases distinguished and described as a megasporogenesis and megagametogenesis (Schneiz *et al*. 1995;

Christensen *et al*. 1997). In Arabidopsis, during megasporogenesis the diploid megaspore mother cell (MMC) divides meiotically giving rise to the haploid megaspores (Figure 1-2a), whereas during megagametogenesis one of the megaspores develops into mature embryo sac (Figure 1-2b, 2c).

Megasporogenesis in Arabidopsis consists of three major events: the megaspore mother cell generation, the meiosis that leads to four haploid megaspores formation and the megaspore spore selection. At the beginning of this phase, in the ovule, a single cell from the sporophytic tissue forms an archesporial cell that differentiates directly into the diploid megaspore mother cell (MMC). Subsequently, the MMC undergoes meiosis resulting in a tetrad of haploid megaspores. Three out of the four megaspores are non-functional and undergo degeneration. In most species including Arabidopsis, the chalazal-most megaspore survives to form the unicellular female gametophyte (Schneiz *et al*., 1995; Christensen *et al*., 1998; Bajon *et al*., 1999). During the next phase of the embryo sac formation, megagmetogenesis, the three major events are involved, such: a series of mitotic divisions, cellularization of the nuclei and a cell differentiation. The functional megaspore mother cell that remained after megasporogeneis elongates along the micropylar – chalazal axis and at the same time its nucleus undergoes the first of three, rounds of mitosis, forming a two-nucleate female gametophyte with one nucleus at either pole. In the center a vacuole is formed. The second nuclear division leads to a formation of the four-nucleate structure. Finally, the third mitosis takes place leading to the formation of the eight-nucleate syncytium. Shortly after the last mitotic division, the syncytial embryo sac undergoes cellularization and forms the seven-celled *Polygonum* type embryo sac. In the mature embryo sac three cells at the micropylar pole organize as the egg apparatus consisting of the egg cell and two synergids whereas at the opposite chalazal end three antipodal cells are localized. The central part of the embryo sac is occupied by the diploid central cell formed from two nuclei which migrate after the third mitosis towards each other and fuse together (Schniez *et al*., 1995; Christensen *et al*., 1998; Drews and Yadegari, 2004; Drews and Koltunow, 2011).

1.1.2. Male Gametophyte Development

The male gametophyte or pollen grain of angiosperms develops within the anther of the stamen (Scott *et al*., 2004) (Figure 1-1). This process consists of two phases:

microsporogenesis where the haploid microspores are formed and microgametogenesis during which mitotic divisions lead to formation of the mature pollen grain (Figure 1-3).

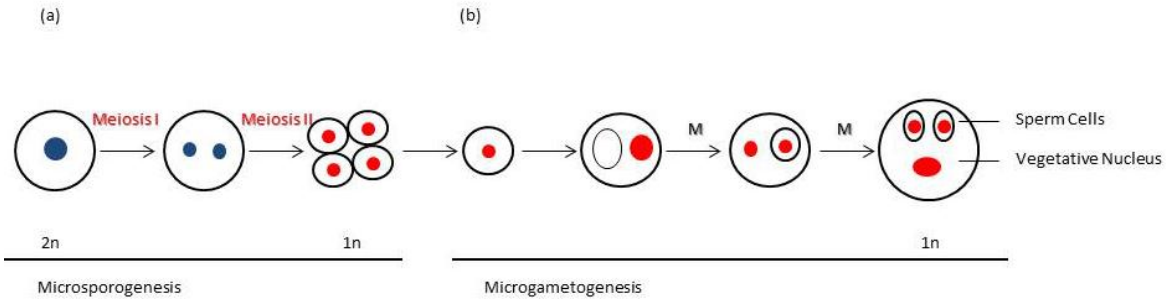


Figure 1-3. Pollen development in Angiosperms. (a) In microsporogenesis a pollen mother cell undergoes meiosis and generates four microspores. (b) Subsequently, during microgametogenesis, each microspore undergoes mitosis resulting in a small generative cell and a large vegetative cell. Finally, the generative cell divides mitotically into two sperm cells. The nuclei in red are reduced (haploid) whereas in blue unreduced (diploid).

At the beginning of microsporogenesis (Figure 1-3), cells of the anther sporophytic tissue differentiate and form the pollen mother cells (PMCs) which after two rounds of meiosis produce a tetrad of haploid microspores (Figure 1-3a). The next step encompasses an asymmetric mitosis of each microspore which results in a production of the bicellular pollen containing two cells of different fate: a vegetative cell and a generative cell (germ cell). The vegetative cell does not undergo any further division. Later during fertilization the vegetative cell forms the pollen tube to deliver sperm cells to the female gametophyte. The generative cell divides once more mitotically to produce the two sperm cells (Figure 1-3b). Usually the second mitotic division occurs during pollen tube growth but in some cases, like in *Arabidopsis thaliana*, it can also take place only before fertilization. Hence, the mature pollen grain consists of two sperm cells and one vegetative cell (McCormick, 1993, 2004; Suzuki, 2009).

1.1.3. Double Fertilization

A characteristic feature of angiosperms is their reproduction mode called double fertilization (Raghavan, 2003). This process was simultaneously discovered in two independent studies more than hundred years ago by Sergius Nawashin and Leon Guignard (Friedman, 1997; Boavida *et al*, 2005). Only recent years brought more information on the molecular mechanisms of the double fertilization (Weterings and

Russell, 2004). Double fertilization comprises a complex mechanism bringing together one sperm with the central cell and another sperm cell with the egg cell leading to the endosperm and the zygote formation, respectively. For a successful fusion of the gametes a series of interactions between the male gametophyte, both the female sporophytic and the gametophytic tissues are required (Hülskamp *et al*., 1995; Johnson and Preuss, 2002; Higashiyama, 2003; Higashiyama and Hamamura, 2008). The interaction starts when the pollen grains are deposited on the carpel's stigma where they germinate, giving rise to a fast growing pollen tube. The pollen tube transports the immotile sperm cells through the female reproductive organs towards the ovules in the ovary (Lord and Russell, 2002). After reaching the ovary the pollen tube exits the transmitting tract and is attracted to the embryo sac. In vitro studies and laser ablation experiment in *Torenia fournieri* revealed an important role of these cells in the pollen tube guidance (Higashiyama *et al*., 2001). Similar results emphasizing an importance of these cells in pollen tube attraction were obtained in *Arabidopsis thaliana*. Furthermore, studies in Arabidopsis indicated also a central cell as an embryo sac structure likely involved in this process (Chen *et al*., 2007). The pollen tube enters the embryo sac through the receptive synergid. Upon entering to the receptive synergid the growth of the pollen tube is ceased and two male gametes are released in a process called a pollen tube discharge (Rotman *et al*., 2003). The molecular and genetic studies revealed several female gametophytic factors that control pollen tube reception (Huck *et al*., 2003; Rotman *et al*., 2003; Escobar-Restrepo *et al.*, 2007; Capron *et al*., 2008; Kessler *et al*., 2010). On the male side only two genes involved in double fertilization were indicated (Boisson-Dernier *et al*., 2009; Miyazaki *et al*., 2009). Shortly, or at the same time after the successful pollen tube discharge the receptive synergid undergoes cell death (Faure *et al.* 2002; Christensen *et al*., 2002). After the rupture, the cytoplasmic content of the pollen tube including the two sperm cells are released in the area between the egg cell and the central cell. Each of the sperm cells can fuse with either female gamete, demonstrating functional equivalency (Hamamura *et al*., 2011).

Recent comparative studies with the reproductive mechanisms in basal angiosperms and their close relatives Gnetales (*Ephedra*) have led to better understanding of the evolutionary origins of this process and have suggested that the double fertilization is inherited from an ancestral of angiosperms in which the second fertilization event gives rise to another embryo that is genetically identical to the to the

normal embryo. The second embryo functions as nourishment for the other and it is suggested that in angiosperms it is modified into the endosperm (Friedman, 1998).

1.2. Apomixis

1.2.1. Definition of Apomixis

Even though the sexual mode of reproduction is a tightly regulated process, some angiosperm plants have developed an alternative mechanism of propagation defined as apomixis. It is an asexual reproduction through seeds where the next generation is produced without a paternal contribution (Nogler, 1984a). Thus, the offspring is genetically identical to the mother and represents a natural type of the clonal propagation. Apomixis occurs frequently in angiosperms. It has been described in more than 400 taxa belonging to more than 40 families of flowering plants (Carman, 1997; Bashaw and Hanna, 1990). It is prevalent within Asteraceae, Rosaceae, Poaceae and improvement of methods determining apomixis might help to include many more taxa to an apomixis group (Bicknell and Koltunow, 2004).

Significantly, the majority of asexual species are facultative apomicts which mean that a proportion of the progeny still is the result of the sexual reproduction. Apomixis often does not completely replace the sexual reproduction but rather those two reproductive pathways coexist in the same plant. This finding and the diversity of apomictic mechanisms lead to the suggestion that apomixis might result from the deregulation and relaxation of the sexual program in time and space. In other words, apomixis can be described as a ”short- circuiting” of the sexual reproduction, where gametes are produced without meiosis and embryo develops prior to fertilization (Grossniklaus *et al*., 1998 Grimanelli *et al*., 2001; Koltunow and Grossniklaus, 2003).

1.2.2. Classification of Apomixis

Despite of the many different ways of apomictic reproduction there are three hallmark steps that distinct this process from the sexual propagation, such: (i) avoidance of meiosis – apomeiosis, (ii) embryo development in absence of fertilization –

parthenogenesis (iii) autonomous endosperm development (Grossniklaus *et al*., 1998; Savidan, 2000; Grossniklaus, 2001) (Figure 1-4).

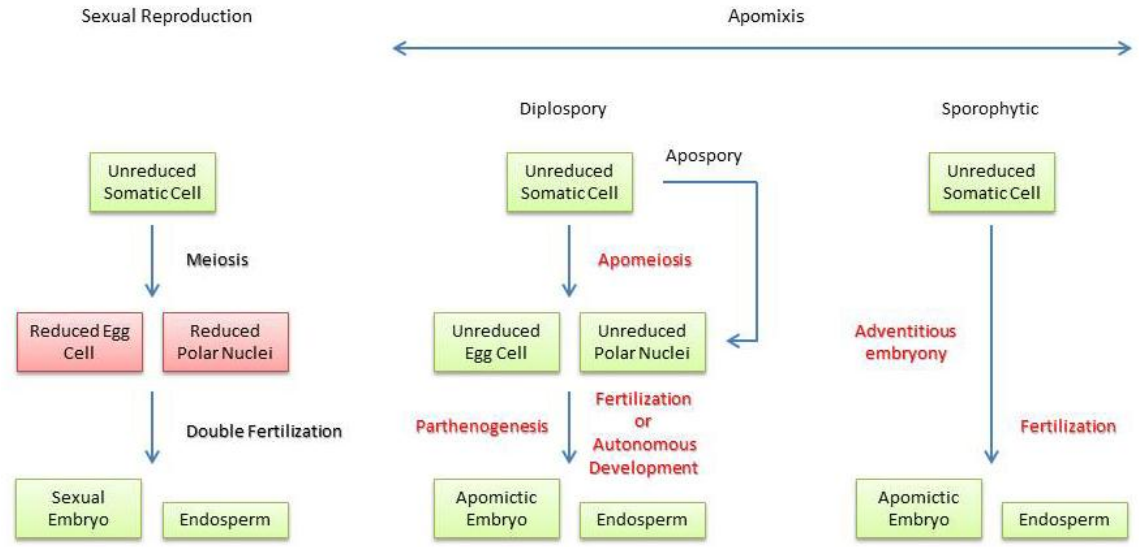


Figure 1-4. Sexual versus asexual mode of reproduction. In sexual species, MMC undergoes meiosis which results in reduced embryo sac development. After fertilization reduced central cell and egg egg cell develop into endosperm and an embryo. Two main types of apomixis are described. In gametophytic apomixis unreduced embryo sac results from avoidance of meiotic reduction. Embryos are formed parthenogenetically and endosperm develops autonomously or requires fertilization. In sporophytic apomixis the embryo sac is formed directly from an unreduced nuclear initial cell. The embryo develops parthenogeneticaly and the endosperm requires fertilization. Red color represents reduced gametophytic stages whereas green unreduced sporophytic stage. Modified form Grossniklaus (2001).

Apomixis is divided into two main classes (Figure 1-5): gametophytic apomixis in which the embryo develops pathenogenetically from the unreduced embryo sac and sporophytic apomixis in which the embryo develops from a somatic cell, usually a nucellar or integumentary cell of the ovule (adventitious embryony). Gametophytic apomixis is further subdivided into two pathways called diplospory and apospory. In the diplosporous type a normal meiosis of the megaspore mother cell either fails and is replaced by a mitotic-like division (mitotic diplospory) or a first division restitution takes place (meiotic diplospory). The diplosporous process leads to a formation of one or two unreduced megaspores (2n). Diplospory is observed in *Antennaria* and *Taraxacum* where the unreduced embryo sacs results from circumvention of meiosis or

a restitution of meiosis I, respectively. Another variation of the diplosporous apomixis exists in *Allium* type with a pre-meiotic additional round of the DNA replication (Grimanelli *et al*., 2001; Grossniklaus, 2001; Bicknell and Koltunow, 2004; van Dijk, 2009).

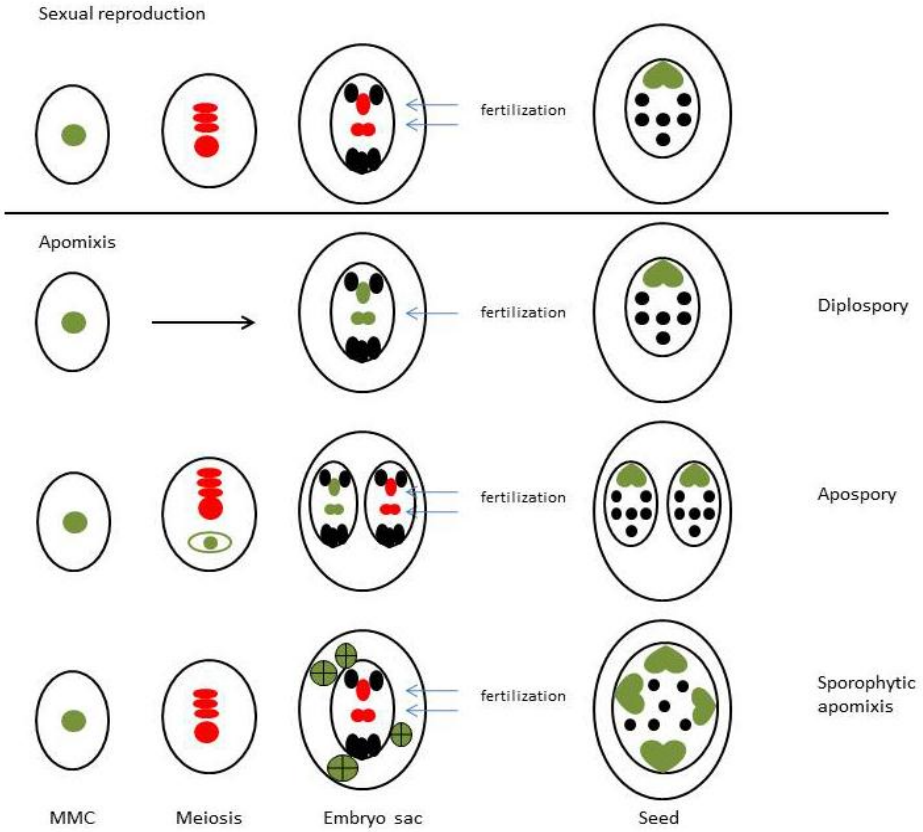


Figure 1-5. Embryo sac and embryo development in sexual and asexual plants. (a) During sexual reproduction, the megaspore mother cell (MMC) undergoes meiosis, resulting in four haploid megaspores. The mature embryo sac contains the haploid egg cell and the binucleate central cell. Double fertilization leads to a diploid embryo and a triploid endosperm formation. (b) In diplospory, either meiosis is omitted or first division restitution takes place resulting in mitosis to produce two diploid spores, one of which survives. In this type of apomixis, the endosperm development requireds fertilization of the central cell, whereas the embryo develops parthenogenetically. (c) In apospory, two embryo sacs co-exist in the ovule. They can originate eigther from haploid functional megaspore or from aposporous initials that develop from somatic nuclear cells next to the MMC. (d) In adventitious embryony or sporophytic apomixis, embryo develops directly from the nucellus or the integument of the ovule. The formation of the embryo sac occurs normally. The nuclei in red are reduced (haploid) whereas in green unreduced (diploid). Modified from Ozias-Akins and van Dijk (2007).

In contrast, in the aposporous type of apomixis the unreduced embryo sac develops from somatic cells of the ovule (aposporus initial). This process might begin at different

stages of the ovule development and often, within the single ovule one or more unreduced embryo sacs (2n) compete with a normal reduced (n), but in most cases the sexually derived gametophyte degenerates. However, a low number of embryos is produced sexually from reduced (n+n) and unreduced (2n+n) embryo sacs is also observed. This type of apomixis is the most common one in higher plants e.i. in the *Pilosella*, *Panicum*, or *Heriacium* genus (Bicknell and Koltunow, 2004).

In sprophytic apomixis the mechanisms are less extensively studied than the gametophytic pathway. It is known that the embryo is not produced parthenogenetically from non-reduced egg cell but it develops directly from somatic cells surrounding the ovule, the nucellus and the inner integument, through mitotic divisions. Thus, a gametophytic phase is missing. in this type of apomixis. The embryo growth requires the endosperm which development is induced by fertilization (pseudogamy). This type of apomixis occurs in *Citrus* species (Koltunow, 1993; van Dijk, Chapter 3, 2009).

1.2.3. Endosperm Development in Apomictic Plants

The *Polygonum*-type embryo sac consists of two haploid polar nuclei that fuse together and form the diploid central cell. Therefore, fertilization of the central cell results in a triploid endosperm that contains two maternal copies and one paternal copy of the genome (Berger, 1999). Disturbances of the maternal: paternal genome contribution in the endosperm of many sexual species often result in seed abortion (Haig and Westoby, 1991). A well illustrated case is maize, where any deviation from the 2m: 1p ratio leads to a failure in endosperm formation and as a consequence seed abortion (Lin B-Y, 1984). In Arabidopsis, however, the endosperm development does not strictly require the 2m: 1p ratio (Scott *et al.*, 1998). Interploidy crosses performed between diploid and tetraploid Arabidopsis plants affected only seed size but not viability. Even though, a moderate imbalance in Arabidopsis is tolerated, the 2m: 1p genome balance is required for the normal seed development.

As in sexual plants, the development of the apomictic embryo is highly dependent on the endosperm formation. In apomictic plants, the endosperm development either requires a paternal contribution (pseudogamous endosperm) or is formed autonomously without fertilization (Koltunow, 1993). In both cases the endosperm develops from an unreduced

central cell while the male gametogenesis is normal. Such a pattern of reproduction disturbs the m: p genome dosage in the apomictic endosperm. For example, in pseudogamous apomicts fusion of the haploid sperm cell with the unreduced central cell is expected to result in endosperm containing 4m: 1p genome ratio. Similarly, in the autonomous endosperm the maternal: paternal genome balance is also disturbed as there is no paternal contribution (4m: 0p). Thus, such m: p imbalance in the apomictic endosperm might result in seed abortion. However, seeds derived asexually rarely show reduction of viability. Apomictic species developed mechanisms that help to circumvent the requirement for the m: p ratio in the endosperm. These mechanisms usually are divided into two main groups (Table 1-1) (Koltunow and Grossniklaus, 2003).

The first group consists of apomicts that modify the fertilization mechanisms in order to maintain the 2m: 1p ratio like in sexual plants. Some apomictic grasses produce an embryo sac containing four diploid nuclei. Such an embryo sac contains only a one polar nucleus which is fertilized by a haploid sperm cell and, thus ensures a 2m: 1p balance after fertilization (Koltunow and Grossniklaus, 2003). Apomictic plants can also adjust the process of the pollen production to maintain required 2m: 1p ratio. *Arabidopsis holboellii* produces unreduced sperm cells that fuse with the unreduced central cell producing 4m: 2p ratio in the endosperm (Naumova *et al*., 2001). Another mechanism of the sperm cells delivery to the embryo sac undergoes modifications in order to ensure genome balance in the apomictic endosperm. For example, in *Dichanthium annulatum* both sperm cells fertilize unfused polar nuclei, resulting in a 4m: 2p ration in the endosperm (Haig and Westoby, 1991).

Species	m: p ratio in endosperm	Fertilization requirement	Applied modification
Sexual			
Polygonum	2m: 1p	Double fertilization	-
Apomict			
Hieracium	4m: 0p	Autonomous endosperm	Insensitive to endosperm balance
Tripsacum	4m: 1p	Pseudogamous 2 unreduced pn x 1 reduced sn	
Panicum	2m: 1p	Pseudogamous 1 unreduced pn x 1 reduced sn	Modified embryo sac developmnet
Arabis	4m: 2p	Pseudogamous 2 unreduced pn x 1 unreduced sn	Modified pollen development
Ranunculus	4m: 2p	Pseudogamous 2 unreduced pn x 2 reduced sn	Modified fertilization mechanism
Dichanthium	2m: 1p	Pseudogamous 2 unreduced pn x 2 reduced sn	

Table 1-1. Modifications of the central cell fertilization resulting in the functional endosperm development in apomictic species. pn- polar nuclei, sn- sperm nuclei. Modified from Koltunow and Grossnikluas (2001)

In the second group, the endosperm development is insensitive to the m: p ratio. To this group belongs *Tripsacum dactyloides* in which the endosperm developed normally, even though, a wide variety of the m: p ratios deviated from 2m: 1p proportion was observed (Grimanelli *et al*., 1997). A similar example of successful seed formation when maternal: paternal ratio in endosperm is imbalanced comes from *Paspalum notatum.* Studies on effects of pollen source and ploidy levels on the endosperm formation in this

species reflected the parental genome ratio in the endosperm ranging from 2m: 1p to 8m: 1p in apomictic plants. Interestingly, in sexual plants of this species the m: p ratio imbalance in the endosperm resulted in seed abortion (Quarin, 1999). However, the most extreme examples of the tolerance for parental genome imbalance were observed in plants such as *Taraxacum* or *Hieracium.* In these species the endosperm is generated autonomously without the paternal contribution (Spielman *et al*., 2003). These results emphasize a tendency towards relaxation of the parental genome ratio requirement in apomicts while such predispositions are not observed in sexual plants.

The dependence of seed development on the maternal: paternal genome dosage can be explained by existence of genomic imprinting present in the endosperm. Genomic imprinting is a process by which genes are expressed in parent-of-origin-specific manner (Haig and Westoby, 1991). It means that some genes are expressed only from the paternally or maternally contributed allele. Hence, adding extra paternal or maternal genomes may lead to disturbance in endosperm development resulting in seed abortion.

1.2.4. Genetic Mechanisms of Apomixis

The genetic control of apomixis remains unclear. It is a heritable trait but it is not known how many genes are involved in this process. The first observations of apomixis were done on *Hieriacium* by Gregor Mendel who was not aware that facultative apomixis occurs in this species. Mendel conducted crosses using *Hieriacium* in order to validate his discoveries on the laws of genetic inheritance but obtained results were very confusing to him. Only 40 year later Mendel's data were explained and associated with apomixis (Koltunow *et al*., 2011).

The genetic analysis of apomixis mainly relies on crosses and recombination events. Recently, improved phenotypic methods and the application of molecular markers have contributed to these studies. However, genetic experiments on apomicts are time consuming and might encounter several complications, caused by: prevalence of polyploidy, epistatic effects, segregation distortions or suppression of recombination (Grossniklaus *et al*., 2001; Ozias-Akins and van Dijk, 2007). In the pioneering studies, inheritance of apomixis in *Ranunculus auricomus* and in *Panicum maximum* (Nogler,

1984b, reviewed in Grimanelli *et al*., 2001; reviewed in Bicknell and Koltunow, 2004) was suggested to be controlled by a single dominant locus. Moreover, apomeiosis and parthenogenesis were co-segregating in these species. Thus, all components of apomixis (apomeiosis, parthenogenesis or endosperm development) seemed to be inherited as the single locus that might be represented by a single master gene, a complex of genes or several tightly linked loci. Similar conclusions were drawn from subsequent experiments performed in other apomictic members of *Poaceae* genus representing aposporous species, such: *Pennisetum squamulatum* (Ozias-Akins *et al*., 1998), *Bracharia decumbens* (Pessino *et al*., 1998), *Paspalum simplex* (Pupilli *et al*. 2001) or diplosporous *Tripsacum dactyloides* (Grimanelli *et al*., 1998). In *Hieracium* inheritance of apospory was also found to be control by the monogenic dominant locus as well (Bicknell *et al*., 2000). In other apomictic plants, however, mapping results suggest that parthenogenesis and apomeiosis are controlled by separate genes, as it was shown in *Poa pratensis* (Barcaccia *et al*., 1998) or in *Erigeron annuus* (Noyes and Rieseberg, 2000). Moreover, later reports from *Poa pratensis* supported this model of inheritance for this species indicating five genes required for apomictic development (Matzk *et al*., 2005). Recent studies performed in *Panicum* showed that three independent loci are involved in apomixis control instead of one locus that was reported previously (Kaushal *et al*., 2008). In *Hieracium*, deletion mapping experiments revealed two independent loci for aposporous and parthenogenesis, named *Loss Of Apospory* (*LOA*) and *Loss Of Parthenogenesis* (*LOP*) (Catanach *et al*., 2006). The polygenic pattern of apomixis inheritance was found in *Taraxacum officinale* where diplospory and parthenogenesis are determined by two single dominant genes: *Diplosporous* (*DIP*) and *Parthenogenesis* (*PAR*) (van Dijk and Bakx-Schatman, 2004).

In some apomictic species, attempts to map elements of apomixis revealed a strong suppression of recombination in these loci. This was found in aposporous grasses, such as *Pennisetum* (Ozias-Akins *et al*., 1998), *Paspalum* (Pupilli *et al*., 2001; Labombarda *et al*., 2002), *Panicum* (Ebina *et al*., 2005) as well as in diplosporous *Tripsacum dactyliodes* (Grimanelli *et al*., 1998). However, there are also apomictic species without suppression of recombination, like in *Bracharia* (Pessino *et al*., 1998), *Poa pratensis* at the locus of parthenogenesis (Matzk *et al*., 2005), *Erigeron annuus* (Noyes and Rieseberg, 2000) or *Taraxacum officinale* (Vijverberg *et al*., 2004; Vijverberg *et al*., 2010).

Recent map-based cloning approaches resulted in preliminary sequences of the apomixis locus. Comparative analysis of the *Paspalum simplex* Apomictic Controlling Locus (ACL) with rice showed rearrangements in this region caused by transposable elements, deletions or single point mutations (Calderini *et al*., 2006). Such rearrangements can probably decrease a capacity of a local chromosome pairing which may result in suppression of recombination. Other studies of BAC sequences from the Apospory-Specific Genomic Region in *Pennisetum squamulatum* selected a putative candidate – *BABY BOOM-like* which might be involved in apomictic pathway (Conner *et al*., 2008). Finally, recent mapping of *Taraxacum officinale* indicated that apomixis locus (200-300Kb) consisits of minimium two genes and possibly includes one or two enhancers or cis-regulatory elements (Vijverberg *et al*., 2010).

In contrast to the genetic control, Carman (1997, 2007) suggested an alternative explanation of apomixis. He presented duplicate-gene asynchrony hypothesis suggesting a role for epigenetic mechanisms in apomixis. It is proposed that asynchronic expression of genes controlling reproductive pathways could be caused by hybridization between genotypes.

1.2.5. Apomixis and Polyploidy

Polyploidy is a result of whole genome duplication (autopolyploid) or hybridization of two or more different but related genomes (allopolyploid). Benefits of polyploidization are based on gene redundancy which improves organism fitness by preservation some gene copies from natural selection and allowing accumulation of new mutations.

In animals the majority of polyploids are parthenogenetic, whereas only a small fraction of polyploid plants is apomictic. In fact, more than 99 % of the plant polyploids are sexual (van Dijk, 2009). But almost all gametophytic apomicts, irrespective of the mechanism, were found to be polyploids, whereas, sexual members of the same or closely related species are usually diploid (Asker and Jerling, 1992). To explain this relationship between the ploidy level and the reproduction mode, it was proposed, that the optimal expression of apomixis might be enhanced by polyploidization (Quarin *et al*., 2001) even though, some rare diploid apomicts were reported (Kojima and Nagato,

1997; Naumowva *et al*., 1999; Koltunow *et al*., 2000). However, in these diploid examples, apomictic seed production was low suggesting again that polyploidy may enhance apomixis expression but is not strictly required for asexual seed formation (Bicknell and Koltunow, 2004). Changes of ploidy status affecting methylation and expression of different alleles might facilitate apomixis in polyploids.

Apomixis was induced in previously diploid plants upon chromosome doubling, but, plants used in this experiment were sexual relatives of apomicts (Quarin *et al*., 2001). Reports describing the reverse experiments of sexual plants recovered form apomicts after the doubling of an apomictic biotype of *Potentilla argentea* (Asker, 1967; reviewed in Bicknell and Koltunow, 2004). Finally, it is also known that the induction of polyploidy in plants rarely resulted in apomictic development. Yet another explanation of the prevalence of polyploidy in apomicts is the hybridization-derived floral asynchrony theory (Carman, 1997, 2007). This hypothesis suggests that apomixis results from the combination of polyploidy and hybridity which lead to asynchronous expression of genes involved in plant sexual reproduction steps. For example, apomixis may arise upon hybridization of divergent ecotypes different in start times and rates of megasprogenesis, megagametogenesis or fertilization.

It was also proposed that supernumerary chromosomes (B-chromosomes) may be involved in apomictic development as a result of intraspecific hybridization (Roche *et al*., 2001). This conclusion may be supported by several facts. These "free" B-chromosomes were found in some apomictic species. Supernumerary chromatin is present mainly in cross-pollinated species and gametophytic apomicts are also associated with cross-pollinating breeding systems. The lack of recombination around the diplospry locus in Tripsacum and Erigeron and the anomalies of meiotic and post-meiotic events could be explained by the supernumerary nature of those genomic regions (reviewed in Roche *et al*., 2001).

The inheritance of apomixis strongly depends on diploid or polyploid gametes. As it was observed in *Ranunculus auricomus*, a dominant allele conferring apomixis could be transmitted only by a diploid gamete. Haploid gametes were also observed in this plant. But, they were producing only sexual progeny suggesting that dominant apomixis allele is gamete-lethal (Nogler, 1984b, 1986). Similar data were obtained in *Hieriacium* in which lethality associated with the transfer of apomixis was observed at the zygotic

stage (Bicknell *et al*., 2000). Moreover, apomixis might be determined by two or more independent loci, such as in Erigeron (Noyes and Riesberg, 2000) or Taraxacum (van Dijk *et al*., 2004). Thus, the transfer of two independent loci to the next generation could be achieved by a production of unreduced gametes. Such a mode of inheritance results in an increased ploidy in the progeny compared to the parents.

1.2.6. Apomixis in Agriculture

Apomixis is wide spread in angiosperms but its prevalence in the agronomical important crops is low. Apomixis in crops was described mainly for perennial forage grasses, *Paspalum*, *Panicum, Cenchrus*, or horticultural plants like *Citrus*, apple, mango and also orchids (Bashaw and Hanna, 1990; Asker and Jerling, 1992; Carman 1995). However, apomictic relatives were also found in wild relatives of important crops, as millet, wheat, rice or maize (Asker and Jerling, 1992; Spillane *et al*. 2001).

Apomixis represents a natural cloning system and is a trait of great interest for plant breeders. Its potential could be utilized in the production of hybrids and fixation of heterosis or any agronomical desirable genotype because in contrast to sexual reproduction it prevents the recombination and therefore the genetic segregation in the next generation. Thus, it assures absolute crop uniformity. Apomixis could reduce other problems arising from sexual reproduction, such as pollinators or cross-compatibility. The trait also drastically reduces the time and costs of hybrid seed production by a simplification of complex crossing schemes and ensuring seeds uniformity. An apomictic propagation of crops through seed could also result in a reduction of the viral transfer associated with a vegetative propagation of such crops like potatos (Jefferson, 1994; Koltunow *et al.*, 1995; Bicknell and Bicknell, 1999; Spillane *et al.*, 2001).

All these reasons led to numerous attempts to introduce apomixis into crop species. Two major approaches where taken. First, exploiting traditional breeding methods in which apomixis is introduced via back-crossing from close apomictic wild relative to sexual crop. Unfortunately, this approach results in high degree of the seed abortion, likely due to gene dosage imbalance. The second strategy takes advantage of genetic manipulation of genes involved in apomictic pathways. However, this method

requires extensive knowledge of the gene functions involved in the developmental pathways both in apomicts and sexual species (Spillane *et al*., 2004).

Although benefits of apomixis technology in crop production are unquestionable there are also some concerns to address, especially about its potential negative impact on our environment. Those problems are primarily the "escape" of apomixis via a direct introgression of the dominant apomixis transgene into related outcrossing species with omitting evolutionary processes. This might cause a reduction of the genetic diversity in crops or in wild relatives and displacement of the sexual development (van Dijk and van Damme, 2000).

1.3. Arabidopsis and Maize in Apomixis Research

In addition to natural apomictic species, Arabidopsis and maize were employed in studies on the mechanisms of apomixis. There are several mutants reported in both Arabidopsis and maize that showed elements of apomxis development. However, there have been no full apomictic mutants recovered from sexual species today. Nevertheless, advantages of model organisms such Arabidopsis may help to shed light on the mechanisms that control the sexual and apomictic pathways.

1.3.1. Apomeiosis

In *Arabidopsis thaliana* the *Polygonum*-type of the embryo sac develops from the haploid megaspore that originates from meiotic divisions of the single cell within the ovule. The megaspore subsequently undergoes three rounds of mitosis and finally the seven-cell embryo sac containing haploid gametes is formed (Schneitz *et al*., 1995). Studies in Arabidopsis identified mutants of the *DYAD/SWITCH1* gene (Siddiqi *et al*., 2000; Mercier *et al*., 2001) that showed defects in female meiosis progression during megasporogenesis. The *DYAD/SWITCH1* is involved in the regulation of the meiotic chromosome organization. Mutations in this gene led to a switch from meiotic to mitotic division of the megaspore mother cell. Such disturbances during meiosis resulted in female sterility, however, a few fertile ovules in the *dyad* mutant were also found.

Moreover, these ovules contained unreduced embryo sacs that resembled the apomeiotic embryo sac of the apomictic plant. Thus, the disruption of the single gene, in a sexual plant resulted in functional apomeiosis, which is a key component of apomixis. However, the *dyad* mutant is incompletely penetrant and produces only a few unreduced embryo sacs (Ravi *et al*., 2008). In contrast to *dyad*, the triple mutant called *MiMe* ("mitosis instead meiosis") in which meiosis is totally replaced by mitosis is fully penetrant. In this mutant the replacement of meiosis with the mitotic division also resulted in the apomeiotic embryo sac production. As a consequence, the *MiMe* plants produce diploid gametes that are genetically identical to the mother. This phenotype was created by combining mutations of three genes: (i) *Osd1* (*omitting second meiosis1*) – omits the second meiotic division, (ii) *Atspo11-1* – eliminates recombination and pairing, (iii) *Atrec8* – modifies chromatid segregation. The three genes conferring the *MiMe* phenotype show strong conservation among plants. This suggests that apomeiosis might be induced in many plant species, including crops, by manipulation of these genes (d`Erfurth *et al*., 2009).

Recent results from the *Arabidopsis thaliana ARGONAUTE9* (*AGO9*) gene suggested that epigenetic regulations may play a role in the plant reproductive development and in differentiation between apomixis and sexuality (Grimanelli, 2012). The *AGO9* gene belongs to *ARGONAUTE* family of genes that are key components of RNA silencing complexes. In the *ago9* mutant the archesporial cell does not undergo meiosis but directly differentiates into a functional megaspore and, the transition from sporophytic to reproductive fate is short-circuited. Consequently, diploid embryo sacs demonstrating aposprous-like phenotype are formed. These results suggest that many plants have the ability to reproduce asexually, but that potential may be suppressed by factors like *AGO9* (Olmedo-Monfil *et al*., 2010).

Genetic screens performed in maize in order to dissect mechanisms of apomixis led to identification of mutants mimicking some aspects of apomictic development. To this group of mutants belongs *elongate* (*el*). Mutants homozygous for the recessive *elongate* gene produce reduced and unreduced eggs at varying proportion. Diploid eggs of this mutant originated from chromosome doubling at the second meiotic division (Rhoades and Dempsey, 1966). Another maize mutant called *Dominant non-reduction4* (*Dnr4*) forms viable unreduced female gametophyte and its phenotype strongly resembled diplospory. It was shown that the phenotype of *Dnr4* results from defects in

chromatin condensation during meiosis of the megaspore mother cell and subsequently fails to segregate chromosomes. The *Dnr4* encodes a protein belonging to the ARGONAUTE protein family, AGO104 and is functionally related to the *Arabidopsis thaliana* ARGONAUTE9 (AGO9). The AGO104 protein is known to be specifically accumulated during sporogenesis in the ovaries and anthers at the time of meiosis. Furthermore, AGO104 was found to be involved in gene silencing via methylation. Thus, maize AGO104 may play a role in the epigenetic regulation of gene expression in the ovule during sexual reproduction. The embryo sac of the *ago104* mutant resembles diplospory, the type of apomixis found in the maize relative *Tripsacum dactyloides*. Interestingly, the *AGO104* locus was located on the maize chromosome 6 in a region which is syntenic to the *Tripsacum* apomixis locus (Sigh *et al*., 2011). The loss-of-function of two maize DNA methyltransferases *DMT102* and *DMT103* results in phenotypes reminiscent of *AGO9* mutant in Arabidopsis. Inactivation of these genes induces production of unreduced gametes and formation of multiple embryo sacs in the ovule. Comparative analysis between the sexual maize, *dmt* mutants and apomictic hybrid revealed that the chromatin state in the archesporial tissue of the *dmt102* mimics the chromatin state found in apomicts (Garcia-Aguilar *et al.*, 2010). These results obtained in maize mutants as well Arabidopsis suggest the epigenetic regulation of the apomictic development.

However, to obtain the apomictic plant, in addition to apomeiosis, parthenogenesis should be induced, as well a normal endosperm development. While, mutants such *ago9*, *MiMe* and *dyad* still require fertilization to produce seeds.

1.3.2. Parthenogenesis

During sexual reproduction, the egg cell is released form meiotic arrest in a process called “egg activation”. In both, animals and plants, this developmental event is highly dependent on a dramatic increase of intracellular calcium ions (Antoine *et al*., 2000; Miyazaki and Ito, 2006). In vitro studies in animals demonstrated that an increasing Ca^{2+} ionophore concentration can trigger parthenogenetic embryo development (Uranga *et al*., 1996), but similar experiments in plants were insufficient to trigger parthenogenesis in absence of fertilization (Antoine *et al*. 2001). However, the Arabidopsis egg cell has some potential to develop into the embryo without fertilization,

as was shown in the *multicopy suppressor of ira 1* (*msi1*) mutant. A mutation in the *MSI1* gene can trigger a parthenogenetic embryo and autonomous endosperm development. However, the embryos undergo abortion at early stages of their development (Guitton and Berger, 2005). In contrast to Arabidopsis, Hieracium *MSI1* (*HMSI1*) unlikely triggers the initiation of autonomous seed development in this plant (Rodrigues *et al*., 2010).

1.3.3. Autonomous Endosperm Development

Genetic screens of *Arabidopsis thaliana* resulted in identification of several mutants displaying aspects of the seed development in the absence of fertilization. These mutants are associated to the loss-of-function of the *Polycomb* group complex encoded by members of the *FERTILIZATION INDEPENDENT SEED* (*FIS*) gene class. This complex contains several proteins, such MEDEA (MEA) (Grossniklaus *et al*., 1998), FERTILIZATION INDEPENDENT SEED 2 (FIS2) (Luo *et al*., 2000), FERTILIZATION INDEPENDEDNT ENDOSPERM (FIE) (Ohad *et al*., 1999), MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) (Köhler *et al*., 2003). These genes were found to be expressed in the central cell of the female gametophyte where they are involved in the repression of genes promoting endosperm growth (Chaudhury *et al*, 2001; Hsieh *et al*., 2003). Loss-of-function mutations in any of these genes lead to central cell nucleus division in absence of fertilization, resulting in autonomous diploid endosperm development (Chaudhury *et al*., 1997). However, in most *fis* seeds the endosperm can only progress to the cellularized phase. In these mutants, embryo like structures was formed at low frequency but their growth eventually is arrested at globular stages (Ohad *et al*., 1996).

Interestingly, specific downregulation of *FIE* in sexual *Hieracium* did not result in the autonomous endosperm proliferation but led to seed abortion after cross-pollination. Furthermore, in apomictic *Heracium* specific downregulation of *FIE* inhibited autonomous embryo and endosperm initiation and most autonomous seeds showed defective embryo and endosperm growth (Rodriguez *et al*., 2008).

1.4. Approaches to Study Apomixis

1.4.1. Molecular Mapping

Apomixis components isolated via map-based cloning strategies might be introduced to crop species. To identify both, aposporic and diplosporic loci, several different molecular markers have been employed, like: amplified fragment length polymorphism (AFLPs) in *Erigeron* (Noyes and Riesberg, 2000) or in grasses (Pessino *et al*., 1998; Labombarda *et al*., 2002), random amplified polymorphic DNA (RAPDs) in *Pennisetum* (Ozias-Akins *et al*., 1998), or restriction fragment length polymorphism (RFLPs) in *Cenchrus* (Gustine *et al*., 1997). The majority of the mapping approaches indicate that apomixis is a dominant trait. Furthermore, in some plant species the apomixis loci were located in a genomic region where recombination is suppressed (Vijverberg and van Dijk, 2007). Suppressed recombination reduces the efficiency of map-based cloning strategies and no isogenic lines for apomixis can be obtained. However, using deletion mutagenesis associated with molecular markers might help to overcome this problem (Catanch *et al*., 2006).

1.4.2. Gene Expression Profiling

An alternative method to identify components of apomixis is the comparison of gene expression profiles form sexual and apomictic reproductive tissues. Such analysis revealed a few candidate genes that are differentially expressed between apomictic and sexual individuals. These candidates, however, had no clear function assigned (Chen *et al*., 1999; Pessino *et al*., 2001; Yamada-Akiyama *et al*., 2009; Polegri *et al*., 2010). Experiments carried out in *Poa pratensis* identified a group of genes putatively involved in signaling and trafficking events during sporogenesis, gametogenesis and embryogenesis (Albertini *et al*., 2004). A more ditailed analysis revealed *PpSERK* and *APOSTART* genes may play a role in apomixis in this grass species (Albertini *et al*., 2005).

Recent progress in the Laser-Assisted Microdissection (LAM) technique greatly increased the efficiency of differential gene expression screens. Using LAM it is possible to isolate specific cell types (i.e. egg cell, central cell) and combined with

RNA-Sequencing methods becomes a powerful tool to study a cell specific gene expression profiles (Schmid *et al*., 2012). This method was shown to be useful in experiments performed on female gametophyte cells which are embedded in sporophytic tissues of the ovule (Schmidt *et al*., 2011). The cell-type-specific transcriptome analysis of the Arabidopsis megaspore mother cell identified novel candidate genes that play a role in RNA processing and translational control at early stages of the sexual female gametophyte development. The transcriptome analysis of the Arabidpsis megaspore mother cell can provide insight into mechanisms controlling megasporogenesis. Subsequently, this knowledge may be used for the manipulation of this process towards apomixis.

1.4.3. Loss-of-Function Approach

A classic genetic approach to dissect a genetic pathway is the mutagenesis disrupting a single gene. Using this strategy in *Arabidopsis thaliana*, a number of mutants that exhibit elements of apomixis were idnetified (Grossniklaus *et al*., 1998; Ohad *et al*., 1999 Luo *et al*., 2000; Köhler *et al*., 2003). Nevertheless, the classical loss-of-function screens have some limitations. Using this approach, many mutants may be overlooked since they have no obvious loss-of-function phenotype. Functional redundancy of genes, especially in plant genomes which consist of many duplicated genes or groups of genes hamper this approach (Blanc *et al*., 2000). Similarly, such screens are not suitable to identify genes which encode products that function at multiple stages of the organism life cycle and are early lethal. The second limitation of loss-of-function screens is the existence of alternative metabolic pathways or regulatory networks (Gu *et al.*, 2003). The problem of loss-of-function screens caused by gene redundancy can be circumvented by combining of several loss-of-function mutants in genes of the same family.

1.4.4. Gain-of-Function Approach

Another strategy relies on a gain-of-function approach (Weigel *et al*., 2000; Tani *et al*., 2004). In this approach the mutant phenotype can be achieved either by the ubiquitous ectopic expression of the gene using a constitutive promoter, like the

cauliflower mosaic virus (CaMV) 35S (Odell *et al*., 1985) or only by enhanced activity of the endogenous expression pathway of the gene of interest. In case of *de novo* apomixis engineering, overexpression of potential candidates in the egg cell could result in parthenogenetic embryo development (Curtis and Grossniklaus, 2007). A group of potential candidate genes identified in Arabidopsis includes: *BABY BOOM* (Boutilier *et al*., 2002), *WUSCHEL* (Zuo *et al*., 2002), *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* (*SERK1*) (Schmidt *et al*., 1997), *LEAFY COLTYLEDON 1* (Lotan *et al*., 1998) and *LAEFY COTYLEDON 2* (Stone *et al*., 2001). All of these genes were found to trigger somatic embryogenesis on various tissues when ectopically expressed.

However, ubiquitous and constitutive expression can lead to similar to loss-of-function lethal or sterile effects if a mis-expressed gene plays important role the plant development. This problem can be overcome by using inducible activation-tagging systems allowing temporal control of expression of tagged genes, and even a degree of spatial control (Matsuhara *et al*., 2000; Zuo *et al*., 2000). Restricted expression in tissues-specific manner in plants can be achieved by employing other systems, such as micro-induction system (Pien *et al*., 2001), the pOp/LhG4 transcription factor system (Baroux *et al*., 2005) or glucocorticoid-dependent gene induction (Craft *et al*., 2005). The temporal and spatial control of the gene expression is provided by an ethanol-inducible system (Deveaux *et al*., 2003), but its usefulness is limited by the volatile nature of the inducer causing unwanted gene activation in neighboring plants. The two-component system for tissue specific gene induction developed in our laboratory (Brand *et al*., 2006) allows localized, conditional gene induction within sectors of the plant exposed to inducer. This system was proved to be able to conditionally activate genes in the Arabidopsis reproductive tissues. Thus, it could be useful for gain-of-function screen for elements of apomixis.

1.5. Aims of the Thesis

The aim of this thesis was to shed light on apomictic processes, focusing on parthenogenesis. The main goal was an attempt to activate the Arabidopsis egg cell to develop into an embryo without fertilization. As apomixis displays a deregulation of the

sexual reproduction in time and space, and is inherited as a dominant trait we performed a gain-of-function screen to engineer parthenogenesis in Arabidopsis. We used the inducible two component XVE system (Brand *et al*., 2006) (Figure 1-6) to randomly mis-express genes in the egg apparatus of the *Arabidopsis thaliana* male sterile mutant in order to induce the egg activation. The two component system provides reliable and conditional gene activation in restricted tissues and cell types. This system relies of chemically inducible chimeric transcription factor XVE (Zuo *et al*., 2000) and its *lexA* binding domain (Figure 1-6). The egg apparatus specific enhancer (*AtEASE*) (Yang *et al*., 2005) allows to restrict the XVE expression to the egg apparatus. Upon induction with human estrogen XVE binds to the *lexA* domain and induces expression of adjacent genes.

In addition to the gain-of-function screen we tested capability of candidate genes to trigger parthenogenesis. Based on preliminary results obtained in our lab (L. Brand, PhD thesis, 2007), mis-expression of the gene encoding WUSCHEL (WUS) in *Arabidopsis thaliana* was performed. Ectopic expression of the *WUS* gene (Zuo *et al*., 2002) is known to induce somatic embryo development in the roots. Preliminary results suggested that siliques of plant lines inducibly expressing *WUS* can elongate upon application of estradiol, prior to anther dehiscence.

We also verified genes involved in the G1/S phase of the plant cell cycle as potential candidates that can trigger cell division and initiate embryogenesis. The Retinoblastoma Related Protein 1 (RBR1) is a key regulator of G1/S phase transition in plants and animals (Ebel *et al.*, 2004). By binding to E2F/DP transcription factors, RBR1 inhibits progression of the G1/S phase arresting a cell in the G1 phase. It has been suggested that RBR1 acts on the egg cell to keep it in the G1 (Sundaresan and Alandete-Saez, 2010) to prevent the progression to the S phase and subsequently completing the cell cycle which could lead to the egg cell activation. Hence, we selected a set of the cell cycle genes important for the RBR1 suppression in order to test whether the mis-expression of these genes in the egg apparatus of the Arabidopsis male sterile mutant will result in the egg cell activation. Moreover, we also decided to directly silence the *RBR1* gene by using an artificial microRNA.

In addition to *RBR1*, the Arabidopsis *MSI1* gene is also suggested to prevent the egg cell activation before fertilization (Sundaresan and Alandete-Saez, 2010). This

hypothesis was supported by observation of parthenogenetic embryos in *msi1* knockout mutants (Guitton and Berger, 2005). However, these embryos abort early suggesting that the *MSI1* gene is also required for the embryo growth. An artificial microRNA (amiRNA) (Schwab *et al*., 2006) was designed to silence the *MSI1* transcript. The expression of amiRNA against *MSI1* was restricted only to the Arabidopsis egg apparatus to avoid affecting MSI1 functions after fertilization. We hypothesized that the absence of *MSI1* transcript in the egg apparatus may induce the parthenogenetic embryo production. Moreover, the functional MSI1 protein after the embryo initiation should prevent its abortion.

In addition to the experiments described in this thesis, two side projects were carried out. Results of these projects are described in two publications that are attached in the appendix (Appendix, 6.1). During the first project we characterized female gametophytic mutants of *Arabidopsis thaliana* that were identified in a gene trap insertional mutagenesis screen (Brukhin *et al*., 2011). The PhD student was responsible for: (i) determination of the T-DNA location in the genome of two mutants by Inverse-PCR, (ii) designing six primer pairs for genotyping of the T-DNA insertion in identified six mutants; (iii) screening of 31 T-DNA insertion lines in order to confirm phenotype of mutants; (iv) examination of the mature pollen grains of six gametophytic mutants by DAPI staining.

The second project was focused on the role of the cullin-containing ubiquitin ligase that is a component of the ubiquitin proteasome pathway in developmental processes in plants (Dumbliauskas *et al*., 2011). The contribution to this project was microscopic examinations of the *cul4* homozygous mutant seed at different developmental stages.

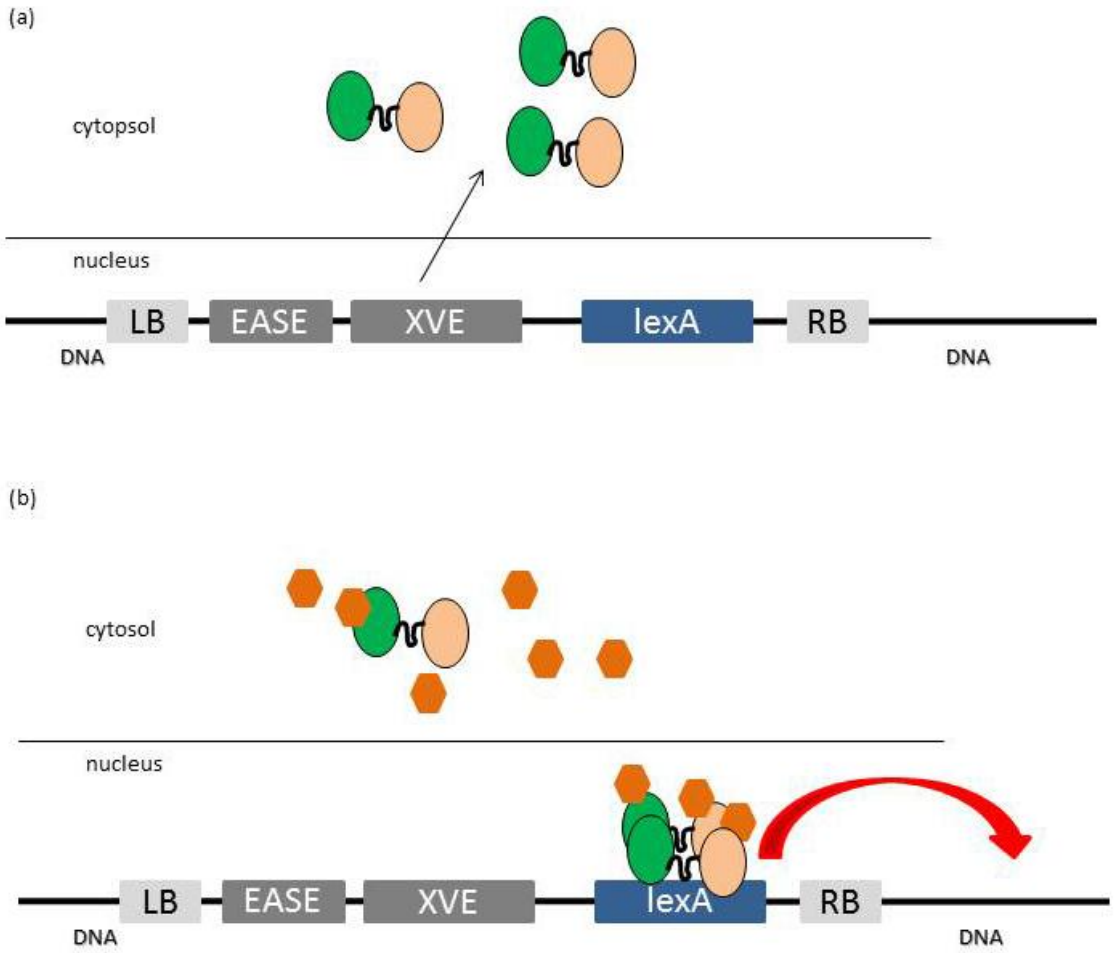


Figure 1-6. The XVE inducible system. The chimeric transcription factor XVE consists of three components: the DNA-binding domain of the bacterial repressor LexA (green), the transactivating domain of VP16 (black), the human estrogen receptor regulatory region (beige). (a) In the absence of inducer, XVE is restricted to cytosol. (b) Upon induction with 17-*ß*-estradiol XVE dimerises and translocates to the nucleus. In the nucleus XVE binds to the LexA target sequences. The LexA consists of eight copies of the LexA operator fused to the *-46min35S* promoter to activate adjacent genes. The VP16 domain recruits the transcription machinery to the *OlexA-TATA.*

2. MATERIAL AND METHODS

2.1. Plant Material

2.1.1. Wild Type *Arabidopsis thaliana* Strains and Male Sterile Mutants

A*rabidopsis thaliana* (L.) Heynh., accessions Columbia (Col) and Landsberg *erecta* (L*er*) were used as wild type strains. Screens performed in order to induce parthenogenetic embryo development relyed on two tightly regulated, conditionally male-sterile plant lines. The observed mutant phenotype is caused by the disruption of the *ALLENE OXIDE SYNTHASE* (*AOS*) gene (at5g42659) that encodes one of the key enzymes involved in jasmonic acid biosynthesis. Mutant lines exhibit defects in anther dehiscence process and reduced filaments elongation that result in male sterility. The mutation has no effect on the female gametophyte and these plant lines can produce seeds after fertilization with wild-type pollen. The *A. thaliana* Col accession mutant *delayed-dehiscence2-2* (*dde2-2*) (von Malek *et al*., 2002) was identified in an *En1*/*Spm1* transposon-induced mutagenesis (Wisman *et al*., 1998). In this mutant transposon footprint created a BstUI restriction site that allows genotyping using CAPS markers (Appendix, Table 6-1) (L. Brand, PhD theses, 2007). The *A. thaliana* L*er* accession *dde2* mutant (exotic line collection GT27) was generated by exploiting the *Ac*/*Ds* gene trap transposon tagging system (Sundaresan *et al*., 1995) within the EXOTIC consortium of the Fifth Framework program of the EU. The *Ds* element contains a kanamycin resistance gene (*nptII*) allowing the selection on MS medium with the appropriate antibiotic.

In both mutant lines pollen production resulting in normal seed set can be restored by spraying the flowering plants daily with 0.01% (v/v) Methyl-Jasmonate ($C_{13}H_{20}O_3$) solution (pure Methyl Jasmonate, Serva Electrophoresis GmbH, Heidelberg, Germany).

2.1.2. Activator Line for Gain-of-Function Screen

The LB122 line was developed as an activator line in the conditional male-sterile *dde2-2* Col background for the inducible activation tagging screen for elements of parthenogenesis. This line contains the activator construct pLB12-*AtEASE* (Figure 2-1) controlling expression of the XVE transcription factor. In addition an *OlexA*-TATA:GUS fragment is present in this vector (Brand *et al*., 2006).

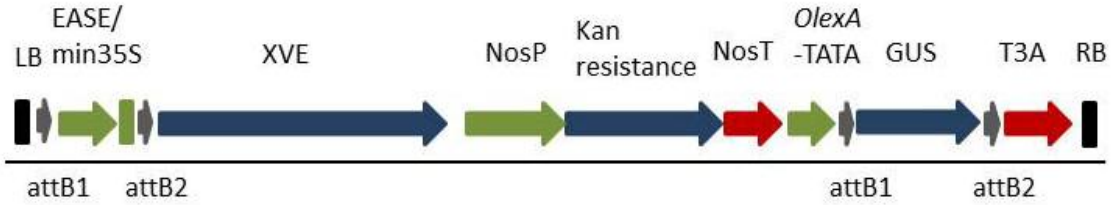


Figure 2-1.Scheme of the T-DNA of the pLB12-*AtEASE* activator vector. The *AtEASE* enhancer is located upstream of the XVE chimeric transcription factor restricting XVE expression to the Arabidopsis egg apparatus. The pLB12-*AtEASE* vector includes an *OlexA*-TATA:GUS unit. It allows verifying the pattern and inducibility of the XVE. This vector contains the kanamycin resistance gene in plants controlled by Nopaline synthase promoter (*nosP*) and terminator (*nosT*).

2.1.3. T-DNA Insertion Lines

The mutant alleles *krp2* and *krp4* correspond to Salk_130744 and Salk_102417 lines (Alonso *et al*., 2003), respectively. Mutant *krp6* is a Sail_548_B03 line (Sessions *et al*., 2002; McElver *et al*., 2001) whereas *krp7* allele is GABI-841D12.03 line (Rosso *et al*., 2003). The T-DNA specific primers were used to confirm the insertion in the genome of mutants (Appendix, Table 6-2).

2.1.4. Plant Growth Conditions

Surface-sterilization of the seeds was done by washing in 70 % EtOH for 10 min followed by Javel treatment for another 10 min (Javel contains 2.5 % sodium hypochlorite, Coop, Switzerland), alternatively 0.01 % Triton X-100 was used. Then the seeds were washed 3-4 times with ddH_2O. Subsequently, the sterile seeds were kept in water to stratify at 4 °C in the cold room for 3-5 days before the seeds were spread on

MS plates. MS medium contained 4.3 g/L Murashige Skoog slats (Carolina Biological Supply Company, Burlington NC, USA), 10 g/L Sucrose, pH 6 (KOH), 10 g/L BactoAgar (Chemie Brunschwig AG, Basel, Switzerland). Antibiotics were added to the medium after autoclaving (50 µg/L kanamycin, AppliChem, Darmstadt, Germany; 15 µg/L hygromycin, Invitrogen Corporation, Carlsbad CA, USA; 5.25 mg/L sulfadiazine, Sigma-Aldrich, St. Louis MO, USA). MS plates with seeds were kept at 22 °C with 14/10 h light/dark cycle in the plant incubator (CU-36L6/D. Percival Scientific Inc., Perry IA, USA) for 14-20 days. The seedlings were then planted in pots containing soil (Einheitserde Classic ED73SM, Einheitserde Werkverband, Sinntal, Jossa, Germany) covered with sand (Quarzsand, Top Quarz M, Top Mineral AG, Wahlen, Switzerland) and watered with 0.6g/L nematodes *Steinernema felitiae* (Andermatt Biocontrol AG, Switzerland) and Solbac (1.2 % Bacillus thuringiensis var. israelensis (12 g/L, Andermatt Biocontrol AG, Grossdietwil, Switzerland). Plants on soil were grown in a greenhouse with 16/8 h light/dark and 22/16 °C temperature cycles at 60 % humidity for the rest of the life cycle. Basta selection was performed on soil by spraying of the plants with 0.05 % (v/v) Basta (Plüss and Stauffer, AG/SA, Oftringen, Switzerland).

2.1.5. Plant Transformation

For plant transformation, the floral dip method was used (Clough and Bent, 1998). The *Agrobacterium thumefaciens* GV3101 bacterium strain containing the required plasmid was grown at 28 °C in a shaker at 180-200 rpm in 5 mL LB medium supplied with gentamycin, rifampicin for the helper plasmid and GV3101 selection and an appropriate antibiotic for plasmid selection. Subsequently, a 250 mL of LB culture containing gentamycin and required antibiotic was inoculated with the pre-culture and was incubated again for 20 h at 28 °C, with shaking at 180-200 rpm. Then the bacteria were pelleted by centrifugation (Beckman centrifuge Avanti J-E, JA-10 rotor, 5000rpm for 10 min in 4 °C; Beckman Coulter, Krefeld, Germany) and resuspended to OD_{600}= 0.8 in transformation medium (5 % w/v sucrose, AppliChem, Darmstadt, Germany) containing 0.02 % v/v Silwet L-77 (Lehle Seeds, Round Rock TX, USA), added before dipping. The flowering plants were transformed as soon as flowers developed. After dipping, the plant pots were laid on their side and covered with a lid for about 24 h.

Afterwards plants were set upright. Conditional male-sterile mutants were daily sprayed with Methyl Jasmonate to allow normal seed set.

2.1.6. Plant Crossing

Plants producing pollen were emasculated 2-3 days before the planed crossing and were pollinated by hand.

There was no need to emasculate *dde2* male-sterile flowers when used as females. To restore the normal pollen production, 3-4 days before the planned crossing the plants were sprayed with Methyl Jasmonate. As soon as mature pollen was observed, stamens were picked for pollination.

2.1.7. Estradiol Induction

The method of estradiol induction depended on the purpose of the experiment. The female gametophyte was induced with solution containing 50 µM 17-*ß*-estradiol (Sigma-Aldrich Inc., St. Luis MO, USA) and rape-seed oil (Schweizer Rapsöl, Coop, Switzerland). A drop of oil containing estradiol was deposited on a plant inflorescence. To mix estradiol with rape-seed oil, a DMSO stock with 20 mM estradiol was used (20 mM estradiol stock in 100 % DMSO). The female gametophyte induction was also carried out by uptake of 20 µM 17-*ß*-estradiol by transpiration through the vasculature tissue. Estradiol induction on the female gametophyte was also performed by regularly dipping inflorescences in estradiol solution (20 µM 17-*ß*-estradiol in H_2O supplied with 0.01 % non-ionic tensides Breakthru S240 (Goldschmidt GmbH, Essen, Germany) that increases the uptake of the inducer through plant tissues). Watering plants on soil (50 µM 17-*ß*-estradiol in tap water) was an alternative and likely inefficient method of induction used. For analysis of plant at the seedling phase, induction using 2 µM 17-*ß*-estradiol in MS plates was carried out (stock solution 20 mM estradiol in 100 % EtOH, added with antibiotics to MS plates).

2.1.8. GUS Staining

For GUS staining floral tissue was harvested and pistils were dissected. Subsequently, the tissue was transferred to the GUS solution [(50 mM Na-phosphate pH 7, 10 mM EDTA, 0.1 % v/v Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 1.5 mg/mL 5-bromo-4-chloro-3-indoxyl- ß-D-glucuronic acid, cyclohexylammonium salt (X-gluc, Biosynth AG, Staad, Switzerland)]. In the next step samples were vacuum infiltrated for about 20 minutes and incubated for about 2 h at 37 °C.

2.1.9. Screening of Transformants

Approximately 9000 individual transformants were screened for gain-of-function phenotypes exhibiting elements of parthenogentic embryo development. The T_1 and T_2 plants were visually assessed for silique elongation 3-5 days after induction with 17-*ß*-estradiol in the male sterile background. The gametophyte induction was carried out by applying a drop of rape-seed oil containing estradiol on the Arabidopsis inflorescence.

2.1.10. Ovules Clearing

For the morphological characterization of ovules, an adaptation of the clearing protocol was used (Yadegari *et al*., 1994). The floral tissue was transferred to an ethanol: acetic acid (9:1) mixture and incubated at 4 °C o/n, followed by washes with 90 % and 70 % EtOH, for 1 h each. The chloral hydrate solution (40 g chloral hydrate, Riedel-DeHaën, Seelze, Germany; 5 mL glycerol anhydrous, Fluka AG, Buchs, Switzerland) dissolved in 10 mL of ddH_2O was used to clear ovules directly before performing the light microscopy.

2.2. Imaging

2.2.1. Stereomicroscope and Light Microscopy

Tissue for microscopy was dissected using a Leica CLS 100X Stereomicroscope (Leica Microsystems, Wetzlar, Germany).

Specimens were analyzed using a Leica DMR light microscope under DIC Nomarski optics (Leica Microsystems, Wetzlar, Germany). Digital images were made using a Magnafire camera (Optronics, Goleta CA, USA) and the Image Pro Express software (Media Cybernetics, Silver Spring MD, USA).

Digital images obtained from microscopy analysis where further processed by Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose CA, USA).

2.3. Standard Protocols, Enzymes and Kits

Standard molecular procedures were carried out as described in Sambrook and Russell (2001) whereas special kit set were used according to manufacturer's recommendations. Chemicals were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless specified otherwise. Restriction enzymes and the corresponding reaction buffers were purchased from New England Biolabs (Ipswich MA, USA). Ligation reactions were performed using T4 DNA ligase from Takara (Takara Ligation Mix, Takara Bio Inc., Otsu, Japan). Taq DNA polymerase and appropriate buffer used for artificial micro RNA product amplification was obtained from Takara (*ExTaq*™ DNA Polymerase, Takara Bio Inc., Otsu, Japan). Tail-PCR reactions were carried out using Taq DNA polymerase (Sigma-Aldrich Inc., St. Luis MO). The cDNA of G1/S genes was amplified with Phusion polymerase, supplied HF buffer and DMSO (Phusion® High-Fidelity DNA Polymerase, New England Biolabs Inc., Ipswich MA, USA) and all PCR reaction were performed according to the instructions of the manufacturer. DNA purification for cloning and PCR product purification from agarose gels was performed by using the NucleoSpin® Extract II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The Qiagen PCR cloning kit was used for direct cloning of PCR products

(Qiagen, Düseldorf, Germany). Large scale plasmid extractions were carried out with the JetStar 2.0 Plasmid Midiprep Kit (Genomed, Chemie Brunschwig AG, Basel, Switzerland).

The Gateway™ cloning system from Invitrogen (Carlsbad CA, USA) was used to perform BP and LR reactions according to the manufacturer requirements with two modifications, only half of the amount of the clonase enzyme was used and a longer incubation time was applied (overnight).

2.3.1. Bacterial Strains, Media, and Transformation

Two strains of chemo-competent *Escherichia coli*, DH5α™ (F^- *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) $_{sup}$E44 λ^- *thi*-1 *gyr*A96 *rel*A1) and DB3.1™ (F^- *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *sup*E44 ara14 *ga*/K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 Δ*leu mtl*1) were used for cloning and plasmid propagation. Transformation of both *E. coli* strains was performed following the calcium chloride (heat-shock) method. Bacteria were stored at -80 °C. Propagation of *E. coli* was performed at 37 °C in *Luria Bertani* (LB) liquid medium or on LB-plates (LB medium and 1.8 % w/v Bacto Agar, Chemie Brunschwig AG, Basel, Switzerland) supplied with the required antibiotics (25 µg/mL chloramphenicol; 10 µg/mL gentamycin sulfate, Fluka AG, Buchs, Switzerland; 50 µg/mL kanamycin, AppliChem, Darmstadt, Germany; 100 µg/mL spectinomycin, Duchefa Biochemie RV, Haarlem, Netherlands).

The *Agrobactrium thumefaciens* strain GV310 containing the helper plasmid pMP90 was used for plant transformation (Koncz and Schell, 1986). The *A. thumefaciens* strain contains gentamycin (40 µg/mL gentamycin sulfate, Fluka AG, Buchs, Switzerland) and rifampicin (50 µg/mL rifampicin) resistance genes. The *A. thumefaciens* competent cells were obtained by growing 25 mL LB liquid culture over night at 28 °C with shaking 180-200 rpm and subsequently were pelleted by centrifugation at 4000 rcf for 10 min. The obtained pellet was re-suspended in 1 mL ice-cold 20 mM $CaCl_2$ and 50 µL aliquots were frozen in liquid nitrogen and stored at -80 °C. The *A. tumefaciens* transformation was performed by adding 1-2 µg of plasmid DNA to frozen competent cells and incubated for 5 min at 37 °C. Subsequently, 200 µL of LB medium was added and samples were further incubation at 28 °C for 2-4 h. After incubation bacteria were

spread on LB plates containing appropriate antibiotics and kept at 28 °C for 3 days in the bacteria incubator. After incubation colonies were harvested and grown in 5 mL LB liquid culture followed by the plasmid mini-prep. Subsequently, the isolated plasmid was control analyzed by restriction digest or sequencing.

All small scale plasmid extraction (mini-preps) was carried out by alkaline lysis method from 1 mL of culture and later dissolved and stored in 20 µL of TE buffer pH 8.0. Bacteria containing plasmids were stored at -80 °C in 10 % glycerol (glycerol anhydrous, Fluka AG, Buchs, Switzerland).

2.3.2. PCR and Sequencing

Standard PCR reactions using Taq polymerase were carried out using a PTC-200 thermocycler (MJ Reasearch, Waltham MA, USA) or S1000^{TM} thermocycler (BioRad, Laboratories, Hercules, CA, USA). The standard PCR reaction was performed at 94 °C for the initial 3 min denaturation step, followed by 29-37 cycles of 15 sec denaturation at 94 °C, annealing temperature (T_a) for 20 sec and elongation at 72 °C. An additional 5 min at 72 °C elongation step followed the last cycle. The annealing temperature was set 2 °C below the melting temperature of the primers as specified by the supplier (Sigma-Aldrich Inc., St. Luis MO, USA). The elongation time was calculated based on an assumed synthesis rate of the one kilobase (kb) amplified product per minute (1 kb min^{-1}). PCR reactions carried out using Phusion polymerase were performed according to manufacturer instructions.

The amplified fragments were separated in 1.2 % agarose gels containing TBE buffer (90 mM tris-borate; 2 mM EDTA) and 0.4 µg mL^{-1} ethidium bromide. Electrophoregrams were visualized under UV light and documented using Gel Doc-It TS^{TM} Imaging system (UVP, Upland, CA, USA)

2.3.3. Tail-PCR

Genomic sequences flanking the T-DNA insertion were identified by thermal asymmetric interlaced (Tail-) PCR (Liu *et al*., 1995). Three nested primers within the left border of the insertion in combination with either the AD1 or the AD2 degenerate

primers were used (Appendix, Table 6-3). The three subsequent PCR reactions were performed as described in Liu *et al.* (1995) with the following modifications. The initial incubation of the first PCR reaction was performed at 94 °C for 2 min. In the secondary and tertiary PCR reactions 15 supercycles and 34 low-stringency cycles were performed, respectively.

The PCR reaction products were purified from an agarose gel and sequenced. Insertion sites were aligned with the library of genomic sequence of *Arabidopsis thaliana* by Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences obtained from Tail-PCR were used to design the T-DNA insertion specific primers. Subsequently, the insertion sites were verified using these specific T-DNA insertions primers and the left border (LB) Tail 1 primer.

All sequencing reactions were performed using a capillary sequencing machine, Hitachi DNA Analyser 3730 (Applied Biosystems, Foster City CA, USA).

2.3.4. Southern Blot

The number of T-DNA genomic insertions present in transformed plant lines was assessed using the Southern blotting technique. Genomic DNA was extracted from leaves. Subsequently, DNA was digested with SacI endonuclease and the obtained fragments separated on 0.8 % agarose gel. Then the separated DNA fragments were transferred on a positively charged nylon membrane (Nylon Membranes positively charged, Roche Diagnostics GmbH, Mannheim, Germany) by the capillary blotting method. Dig probes for pMJ1 and pMJ2 insertions were produced by PCR amplification (Appendix, Table 6-4) to allow incorporation of the anti-digoxigenin-AP Fab fragments (Roche Diagnostics GmbH, Mannheim, Germany) into the probe DNA. Visualization of the probe was performed according to the standard protocol for chemiluminescence using the CDP-star substrate (Boehringer, Mannheim, Germany). Subsequently the nylon membrane was exposed to standard laboratory X-ray film (FUJIFILM, FUJIFILM Corporation, Tokyo, Japan).

2.3.5. Ploidy Analysis

Ploidy measurements were carried out using a Ploidy Analyzer PA-I from Partec (Münster, Germany) according to the manufacturers' instructions. All buffers used for the analysis were supplied by the manufacturer. A piece (~1 cm^2) of leaf tissue was collected from the plant of interest and finely chopped. The measurements were proceeded by a calibration of the polidy analyzer with diploid plant material (WT Col). The gain and the lowest level (L-L) (minimal signed intensity to be scored as real count) were set in accordance to the controls and ranged generally from 350 to 450 for the gain and from 20 to 50 for the L-L. The speed was set at 2 µl/s.

2.4. Vectors

2.4.1. pMDC113

In order to ectopically express the *WUSCHEL* gene pMDC113 was used (Figure 2-2). This construct was derived from an estradiol inducible vector pMDC7 (Curtis and Grossniklaus, 2003) and contains an XVE transcription factor (Zuo *et al*., 2000) under the transcription control of the *GT10-90* promoter and the *OlexA-TATA:WUS* responder unit.

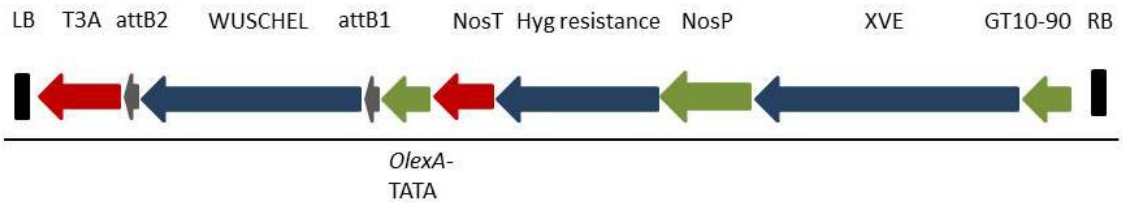


Figure 2-2. Scheme of the pMDC113 vector. This vector was used for condtional mis-expression of the *WUS* gene. The pMDC113 construct contains XVE and the *OlexA-TATA* responder unit. The expression of the XVE is controlled by the *GT10-90* promoter. (LB/RB) left and right borders, (*nosP*) nos promoter, (*hpt*) gene conferring hygromycin resisistance in plants, (*nosT*) nos terminator, (attB/R) Gateway™ recombination sites, a *WUS* gene *CDS* is inserted into the *CDS*- cassette, (*T3A*) terminator for the Gateway™ cassette are the remaining components of the pMDC113 vector.

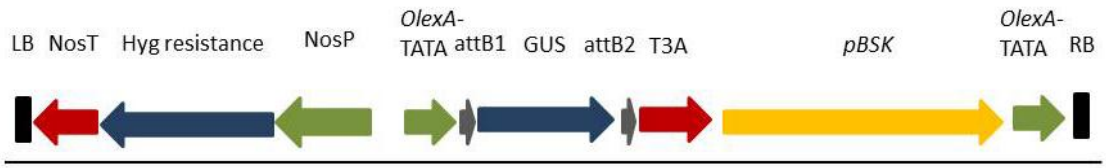
2.4.2. Responder Vectors Used for Gain-of-Function Screen

For the activation tagging screen using the pLB12-*AtEASE* activator, the responder constructs pLB66 and pLB71 were used (Figure 2-3a and b).

Vector pLB66 (Figure 2-3a) contains two *OlexA-TATA* XVE-responsive promoters. One is located upstream of the Gateway™ cloning cassette, the other is adjacent to the right border (RB) sequence of the insertion, so that this vector can be used for conditional activation tagging experiments (Brand *et al*., 2006).

The pLB71 was derived from the pLB66 vector by removing a ~5600 bp XbaI fragment between Nos promoter and RB adjacent *OlexA-TATA* cassette. In the new responder vector the fragment between left and right border consists of the hygromycin resistance genes with its promoter and terminator and one *OlexA-TATA* cassette adjacent to the RB (Figure 2-3b).

(a)



(b)

Figure 2-3. The T-DNA of responder vectors.
(a) The pLB66 vector contains two *OlexA*-TATA units. (LB/RB) left and right border, (*nosP*) nos promoter, (*hpt*) gene conferring hygromycin resisistance in plants, (*nosT*) nos terminator, (attB/R) Gateway™ recombination sites, a *GFP* reporter gene *CDS* is inserted into the *CDS*- cassette, (*T3A*) terminator are components of the pLB66 vector. Further, this vector contains the pBluescriptSK sequence (*pBSK*), providing a bacterial origin of replication (*ColE1 ori*) and ampicillin resistance for plasmid resque.
(b) The pLB71 T-DNA. Between (LB/RB) left and right borders of the pLB71 T-DNA a (*nptII*) gene conferring kanamycin resistance in plants, (*nosP*) nos promoter, (*nosT*) nos terminator and the OlexA-TATA unit are present.

2.4.3. Construction of pMJ1 and pMJ2 Vectors

The pMJ1 and pMJ2 vectors were developed in order to mis-express candidate genes in the *Arabidopsis thaliana* egg apparatus under control of the *AtEASE*/min35S promoter (Figure 2-4).

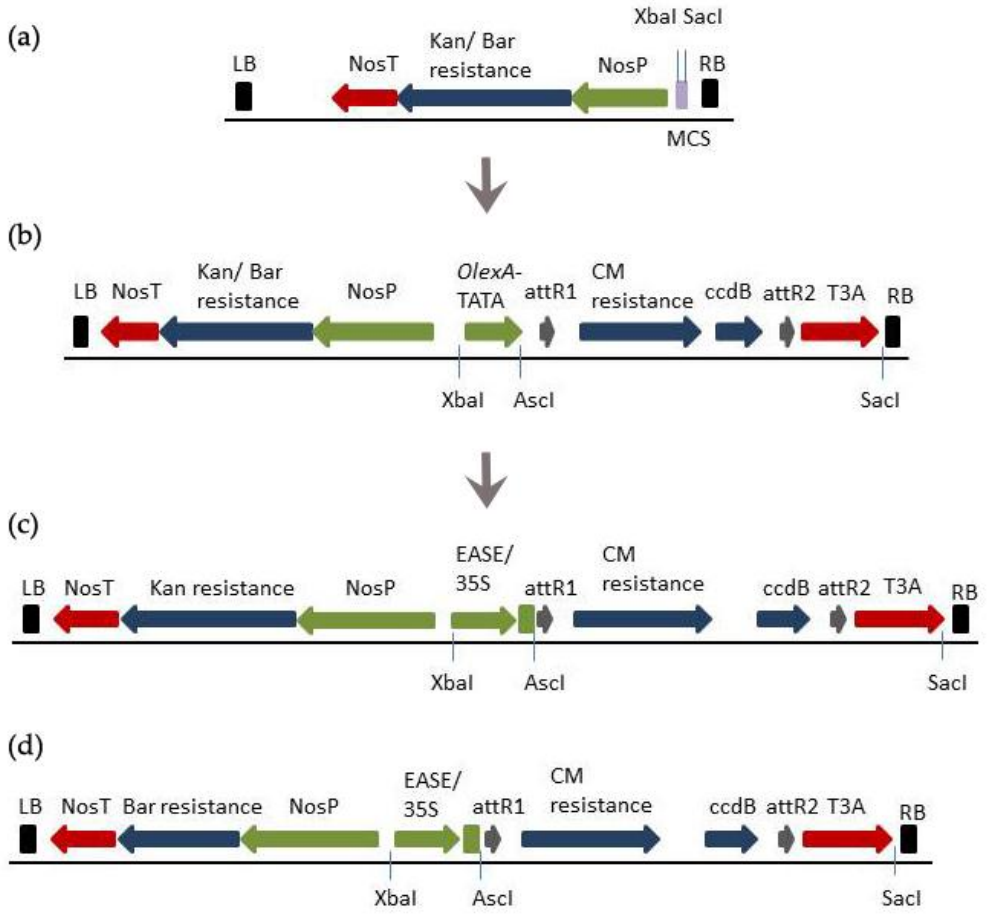


Figure 2-4. Generation of the pMJ1 and pMJ2 expression vectors.
(a) The T-DNA of pMoa binary vectors. The pMoa series confer the spectinomycin resistance in bacteria and provide different markers for plant selection. These markers for plant selection are controlled by the Nopaline synthase promoter (*nosP*) and terminator (*nosT*). pMoa33 confers kanamycin resistance (*kan*^{r}) and pMoa36 basta (*bar*^{r}) resistance, respectively. MCS- multiple cloning site, LB- left border, RB- right border.
(b) The T-DNA of pHA1 and pQAN1 vectors contains the OlexA-attR1-Cmr-ccdB-attR2 fragment and a marker gene for selection in plants. pHA1 confers kanamycin resistance (*kan*^{r}) and pHA1 basta (*bar*^{r}) resistance, respectively.
(c) pMJ1 and (d) pMJ2. Both vectors contain the GatewayTM cassette consisting of (attB) GatewayTM recombination sites, (CMr) chloramphenicol resistance in bacteria and (ccdB) gene. Upstream of the Gateway unit, the At EASE/ -46min35S promoter was cloned. T-DNA of these vectors contains genes conferring a kanamycin (*nptII*) and basta resistance (*bar*^{r}) in plants, respectively. The transcription of selectable marker genes is controlled by Nopaline synthase promoter (*nosP*) and terminator (*nosT*). These vectors confer spectinomycin resistance in bacteria.

The pMJ1 and the pMJ2 were constructed based on the pHA1 and the pQAN1 plasmids, respectively. The pHA1 (obtained from Hiroko Asano) and the pQAN1 (obtained from Quy A. Ngo) were derived from minimal T-DNA binary vectors pMoa (Barrell and Connor, 2006). The pMoa vectors have very small T-DNAs with different marker for plant selection and the right border of the T-DNA a multiple cloning site was introduced (Figure 2-4a). pHA1 and pQAN1 were generated by introducing the *OlexA-attR1-Cm^r-ccdB-attR2* fragment into pMoa33 and pMoa36, respectively (Figure 2-4b). The *attR1-Cm^r-ccdB-attR2* is an integration region from the Gateway^{TM} cloning system (Invitrogen) containing *att*-recombination sites flanking the *ccdB* gene (Bernard and Couturier, 1992) for positive selection in the *E. coli* DB3.1 strain and the bacterial chloramphenicol resistance (cm^r) gene. The 3' of the Gateway cassette for coding sequences (CDS) is followed by a T3A terminator. The *attR1-Cm^r-ccdB-attR2* fragment was obtained from the pMDC221 vector and introduced into pMoa33 and pMoa36 by conventional cloning using XbaI and SacI restriction enzymes.

In order to generate pMJ1 and pMJ2 vectors (Figure 2-4c and d) the *AtEASE*/-46min35S promoter was cloned into pHA1 and pQAN1, respectively. The sequence of the *AtEASE* together with 35S minimal promoter was amplified using Phusion polymerase (Phusion® High Fidelity Polymerase, New England Biolabs Inc., Ipswich, UK) from the pWY-093.1 vector (Yang *et al.*, 2005) using primers containing XbaI and AscI restriction site adapters (Appendix, Table 6-5). The amplified fragment was sub-cloned into the pDrive vector (Qiagen PCR Cloning Kit, Qiagen, Düseldorf, Germany), propagated in DH5α competent cells and isolated by alkaline lysis method. Subsequently, pDrive-*AtEASE*/-46min35S and the destination vectors pQAN1 or pHA1 were digested overnight at 37°C using XbaI and AscI restriction enzymes. The digests were heat-inactivated at 65°C for 20 min and subsequently ligated. The resulting plasmids were propagated in DB3.1 cells.

2.4.4. pEntry Vectors

Entry clones were generated by amplifying cDNA of *CDKA;1*, *CDKD;3*, *CycA3;1*, *CycD2;1*, *CycD3;1*, *CycH;1*, *E2Fb* and *DPb* using two set of primers, first set for gene-specific amplification including 12 bases of the *attB1* and *attB2* site and the second to get the complete *attB* sequences of the PCR product (Appendix, Table 6-6).

Products were amplified using Phusion polymerase. Subsequently, the *att*B-PCR products were integrated into pDonor207 using BP clonase and then transfered into pMJ1 or pMJ2 using LR clonase. The resulting vectors were propagated in DH5α and sequenced.

2.5. Generation of Artificial microRNAs

The artificial microRNA (amiRNA) targeting the *RBR1* and *MSI1* genes, respectively, were designed using the artificial microRNA a web designer the Web MicroRNA Designer (WMD) platform (Schwab *et al*., 2006; Ossowski *et al*., 2008). The introduction of the amiRNA-*RBR1* (TACTGTGTGAAATAAGAGCGT) and amiRNA-*MSI1* (TTAGCGTTAATATCCCACGAG) sequences into the *Arabidopsis thaliana* microRNA precursor *MIR319a* was carried out by overlapping PCR using four oligonucleotides for each amiRNA and two generic primers (Appendix, Table 6-7) hybridizing to the pRS300 vector. The pRS300 plasmid used as a PCR template harbors the Arabidopsis endogenous *miR319a* precursor in a pBluescript SK backbone (Schwab *et al*., 2006).

The obtained precursor fragments were cloned into the pDrive plasmid and propagated using DH5α competent cells. Modified amiRNAs were amplified by two sets of PCR primers, first set for the fragment specific amplification including 12 bases of the *att*B1 and *att*B2 site and the second to get the complete attB sequences of the PCR product. The resulting PCR products were introduced by the BP reaction into pDonor207 to create pENTRY vectors. Subsequently, the LR reaction was performed to introduce the amiRNAs into destination vector pMJ1. After sequencing, the expression vectors pMJ1-*amiRNA-RBR1* and pMJ1-*amiRNA*-MSI1 were used to transform Arabidopsis Columbia *dde2-2* male sterile mutant.

3. RESULTS

3.1. Gain-of-Function Screen

Progeny of sexually reproducing flowering plants results from the fusion of the reduced female and male gametes in a process described as double fertilization. During apomictic reproduction, the progeny is produced in the absence of meiosis and without paternal contribution. The mechanism of this process is thought to have evolved from a deregulation of ancestral sexual processes resulting in (i) circumvention of meiosis (apomeiosis); (ii) embryo development without fertilization (parthenogenesis) and (iii) the development of functional endosperm (Koltunow and Grossniklaus, 2003). Since apomixis is a dominant trait, a gain-of-function screen to deregulate gene expression in the egg apparatus in order to induce parthenogenesis was performed. A versatile two-component system providing reliable conditional gene activation in the egg apparatus was employed to express randomly tagged genes in the tissue specific manner (Brand *et al*., 2006). This system comprises of an activator and a responder unit. The activator T-DNA unit consists of the chemically inducible chimeric transcription factor XVE (Zuo *et al*., 2000) with a minimal CaMV 35S promoter under a transcription control of the egg apparatus specific promoter, *AtEASE/*min35S (Yang *et al*., 2005). The responder T-DNA component contains an XVE-responsive promoter, *lexA* operator that can be used to mis-express randomly tagged genes. Upon chemical induction with 17-*ß*-estradiol the chimeric transcription factor XVE bind to its responsive promoter *lexA* and transactivates the adjacent gene. We aimed to generate 9 000 independent transformants and screen for elements of apomixis upon induction in the male sterile background.

The conditionally male sterile LB122 (Brand *et al*., 2006) line containing XVE transcription factor under a transcriptional control of the *AtEASE* promoter was used as the activator line in the screen. Subsequently, this line was super-transformed with the responder vector pLB66 (Brand *et al*., 2006) and double transformants containing both units of the inducible system were generated. On MS plates containing appropriate antibiotics about 5200 double transformants were obtained. Each T_1 line was supposed

to be homozygous for the activator unit and heterozygous for the responder unit and segregate in a 3: 1 ratio in the next T_2 generation. Seven seedlings of each T_2 line showing a segregation ratio of 3: 1 were planted in one pot. During flowering inflorescences of these lines were induced with 17-*ß*-estradiol and screened for gain-of-function mutants that show conditional seed development phenotypes in the absence of fertilization. In order to activate the embryo development in the female gametophyte the chemical inducer needs to enter and efficiently penetrate the egg apparatus of the embryo sac at the micropylar end of the ovule. In case of this screen the chemical induction was carried out by applying a drop of pure rape-seed oil containing 50 µM of 17-*ß*-estradiol. However, there were two other alternative application methods tested, to determine which is the fastest and the most efficient to screen a high number of lines. Estradiol induction was carried out by normal watering with tap water containing estradiol. Alternatively, inflorescences were dipped in estradiol solution. However, both methods, in comparison to rape-seed oil based induction, were found not to be ideal for screening thousands of individuals. Dipping requires regular treatment (3-4 times) of the plant which causes damages and stress to the plants. Induction by watering does not stress the plant, however, it is not possible to maintain induced and un-induced sectors on the plant. Randomly collected transformant' flower samples were checked 2-4 days after rape-seed oil based for GUS activity to assure effectiveness of the chemical induction. The figure 3-1A shows that the induced *GUS* gene expression in the egg apparatus of the ovules can be detected in synergids already one day after the induction with estradiol. Subsequently, the *GUS* expression is observed in the whole egg apparatus (Figure 3-1C). 3-4 days after the induction a diffusion of the *GUS* product is observed in the embryo sac and surrounding tissue (Figure 3-1D).These observation suggested that 50 µM concentration of 17- *ß*-estradiol is sufficient to activate GUS expression.

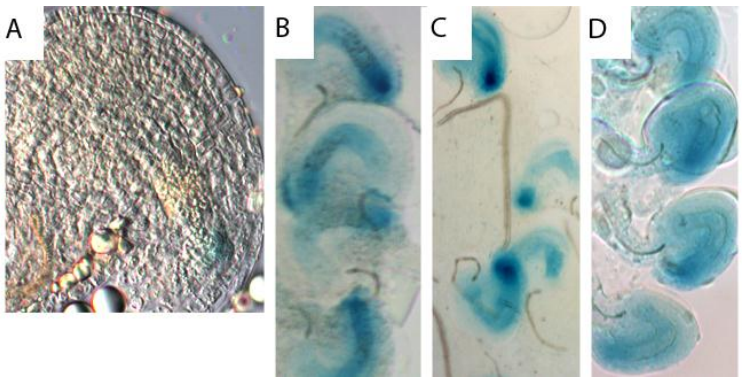


Figure 3-1. Expression of the *GUS* reporter gene in the egg apparatus of the T_1 individuals after induction with 50µM of 17-ß-estradiol. (A) GUS expression 1day after induction (dai). (B) GUS expression 2 dai. (C) GUS expression 3 dai. (D) GUS expression 4dai.

Usually 3-5 days after the induction transformants were screened for silique elongation. From the T_2 generation we isolated 78 candidates. Siliques of selected individuals seemed to be longer on induced inflorescences in comparison to those found on not induced ones (Fig. 3-2). However, in contrast to the wild type, these siliques were thin and not very long suggesting lack of the developing seed inside. Besides the visual assessment, candidates were analyzed by light microscopy. Unfortunately, the estradiol induction method using rape-seed oil generated technical problems regarding pistil dissection and clearing of ovules, the structures of the embryo sac were not easily distinguishable.

Figure 3-2. Length of siliques observed on not induced (A) and induced (B) inflorescences. Some siliques from induced inflorescrnces were longer in comparison to the control, however, no developing seeds were found.

Six lines (468a, 959, 854, 3621, 3615, and 3876) produced WT siliques before application of 17- *ß*-estradiol and were found not to carry *dde2-2* mutation (Figure 3-3).

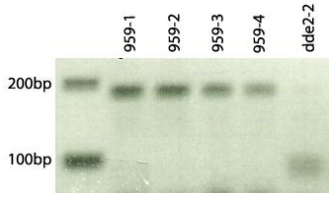


Figure 3-3. The footprint of the dde2-2 mutation created a novel BstU I restriction site, which can be used for genotyping. A cleaved amplified polymorphic sequence (CAPS) marker is not present in wild type plants.
100 bp- CAPS marker; 200- bp wild type marker

The putative mutants isolated in this screen were treated with methyl jasmonate to restore the pollen production and obtain seeds. The T_3 generation was re-screened to confirm heritability of the observed phenotype. Progeny of putative candidates was planted on soil and induced with estradiol diluted in oil. Alternatively, candidates were planted in closed plastic containers containing MS medium with 2 µM 17-*ß*-estradiol. This method helped to overcome problems with dissecting and tissue clearing and

prevented tested individuals from accidental pollination which might occur in the greenhouse where male sterile mutants often grow in vicinity of fertile plants. However, re-screening of candidates did not support the initial results and ovules of induced siliques did not show parthenogenetic embryo development. The observed ovules contained a WT embryo sac without any structures suggesting embryo development (Figure 3-4).

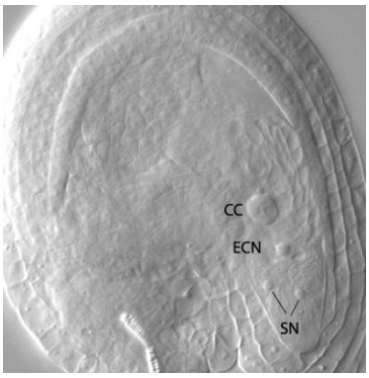


Figure 3-4. The *dde2-2* ovule containing normal embryo sac. CC- central cell, ECN- egg cell nucleus, SN- synergids nuclei.

Among the selected 78 putative candidates, there were two mutant lines (1255 and 4368) producing siliques which were round suggesting developing seeds inside. However, there was only a single longer silique usually containing only one seed which suggested accidental pollination. Nevertheless, even such individuals were further analyzed. Developing embryos were rescued and plated on MS plates without any antibiotic and the maternal plant was sprayed with methyl jasmonate to obtain seeds. Developing seedlings were planted on soil and ploidy level of these lines was checked (Figure 3-5). If these plants resulted from parthenogenetic embryo development they should be haploid like the egg cell. Unfortunately, these plants proved to be diploid confirming that seed development was caused by pollination. Additionally, these candidate lines were induced with estradiol but no silique elongation was observed.

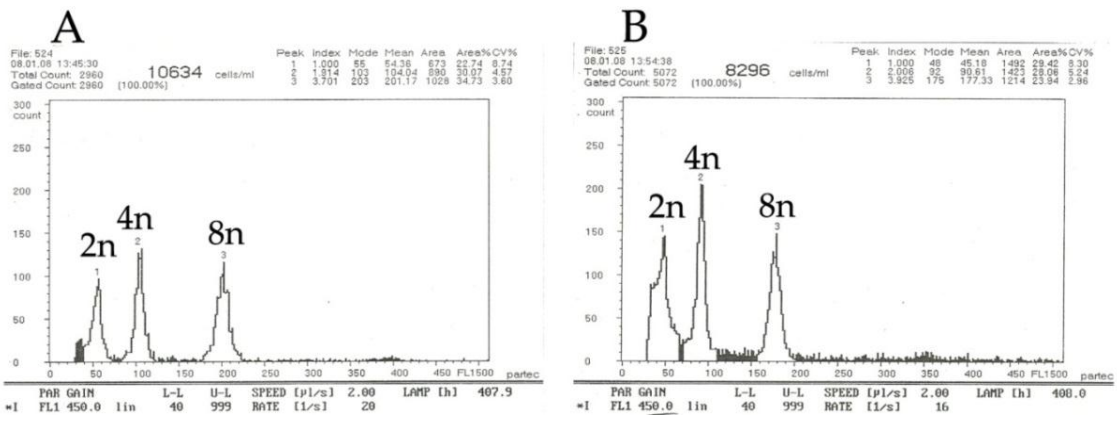


Figure 3-5. Ploidy analysis. (A) Wt diploid plant. (B) Candidate mutant line 1255. On the X-axis size of the nuclei is represented (50=2n, 100=4n, etc) while on the Y-axis represents number of counting for a specific size.

At the right border the responder vector pLB66 contains *OlexA-TATA* fragment which consists of eight copies of the *lexA* operator fused to the -46 min 35S promoter. During our screen, sequencing of the responder pLB66 vector isolated from Agrobacterium competent cells revealed truncations in the *OlexA-TATA* fragment. Such truncation may result from recombination that takes place during bacteria reproduction. Thus, a new modified responder vector pLB71 was constructed. The pLB71 vector was generated from the pLB66 vector by removing a ~5600 bp XbaI- fragment between nos promoter and the right border adjacent the *OlexA-TATA* cassette. The resulting plasmid contains following functional features: the hygromycin resistance gene for plant selection under the transcriptional control of the nos promoter and terminator, respectively, and one *OlexA-TATA* cassette adjacent to the right border for activation tagging. In order to confirm the identity of the *OlexA-TATA* fragment, an amplification of this fragment by PCR was carried out on DNA of mutant plants containing the new tagging pLB71 construct. Primers flanking the *OlexA-TATA*/35S minimal promoter fragment were used to amplify 417 base pairs DNA fragment (Appendix, Table 6-8). For this purpose 10 random mutant plant samples were collected and PCR analyses were carried out revealing that in 9 mutants the analyzed fragment was of expected size while one mutant contained shorter PCR fragment (20-30 bp shorter) (Figure 3-6).

Figure 3-6. Amplification of the *OlexA*-TATA/35S fragment form T-DNA insertion of the pLB71 vector. The red triangular indicates an individual containing shorter *OlexA*-TATA/35S fragment indicating recombination in this part of the T-DNA. The remaining mutant lines contain *OlexA*-TATA/35S fragment of expected size.

Thus, even in more compact modified pLB71 vector recombination still takes place. Nevertheless, screen for gain-of-function phenotype was continued using the activator line LB122 as before and the pLB71 vector as the new responder vector. The last fraction of approximately 4000 mutants was screened in the T_1 generation in order to reduce the time for completing the screen. The chemical induction with 50 µM 17-ß-estradiol was carried out as described. Methyl jasmonte was applied to produce progeny of individuals chosen for the re-screen in the T_2 generation. Out of the 4000, 14 mutants were identified as putative candidates. Five of these lines (1, 2, 3, 4, and 9) produced longer WT-like siliques containing seeds (Figure 3-7).

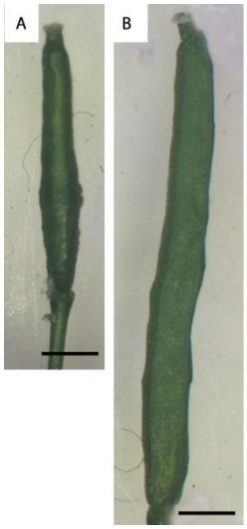


Figure 3-7. (A) Silique containing unfertilized ovules (B) Fertilized silique containing developing seeds.

Suspiciously, such siliques were observed on both induced and un-induced inflorescences. The mutant 1 was analyzed by light microscopy and observation of developing seeds revealed presence of both embryo and endosperm (Figure 3-8). A PCR using diagnostic *dde2-2* primers revealed that this mutant line as well as line 9 does not carry the *dde2-2* mutation. The remaining lines (2, 3, and 4) were re-screened in the next T_2 generation by growing them in closed plastic containers on MS medium containing 2 µM 17-*ß*-estradiol. None of selected putative candidates showed a parthenogenetic phenotype in T_2 generation, there were no developing embryos in the embryo sac observed. This suggested that a seed development observed in these mutants most probably resulted from accidental methyl jasmonate application or pollination.

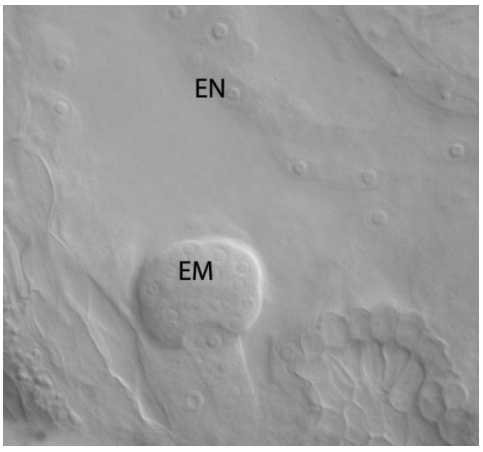


Figure 3-8. Normal seed development observed in the candidate mutant line. EM- embryo, EN- endosperm nucleus.

3.2. Candidate Gene Approach

3.2.1. *WUSCHEL* Ectopic Expression Under Ubiquitous *GT10-90* Promoter

Previous observation carried out on male fertile WT L*er* plants transformed with the T-DNA vector pMDC113 suggested that the presence of 17-*ß*-estradiol might induce siliques elongation prior to anthesis. The pMDC113 vector contains the *XVE* transcription factor under the transcriptional control of the *GT10-90* promoter and the *OlexA-TATA-WUS* responder unit. This vector was generated form pMDC7 (Curtis and Grossniklaus, 2003). Ploidy experiments revealed that plantlets derived from the seed of the induced plant are diploid. Microscopy analysis showed that seeds developing within elongating siliques after induction contain normal embryos most probably without endosperm. However, in the LB122 line ectopic expression of *WUS* in the egg apparatus showed no silique elongation. This finding suggested that embryos observed in *Ler* pMDC113 lines did not originate from the egg cell or that embryo development requires broader expression pattern of WUS (L. Brand, PhD thesis, 2007).

In order to confirm these preliminary results from ectopic *WUSCHEL* expression, the L*er* conditional male sterile *dde2* line (GT27) was transformed with the inducible pMDC113 construct. The seeds of the primary transformants were first selected on MS plates containing appropriate antibiotics. Subsequently, seeds of the T_1 population were plated on MS medium supplied with 17-*ß*-estradiol. Seedlings showing the ectopic *WUS* phenotype by producing somatic embryos on roots in the presence of estradiol were selected to grow on soil (Figure 3-9).

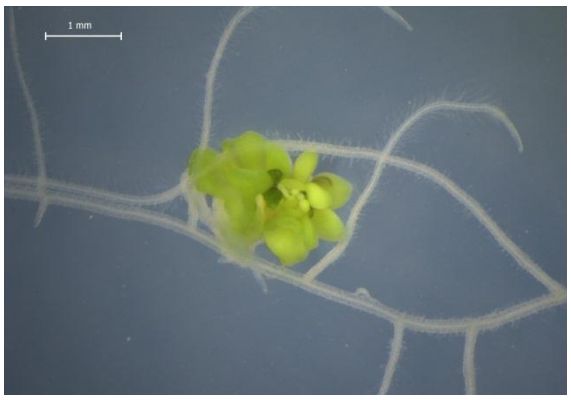


Figure 3-9. Inducible somatic embryo developing on roots of the L*er dde2* line containig the pMDC113 vector. Bar- 1mm.

Inflorescences of mature plant were induced with 17-ß-estradiol using two methods: induction by rape-seed oil application and induction in eppendorf tubes containing 17-ß-estradiol in water. Screening of plants induced with oil containing estradiol did not reveal silique elongation. Floral organs were clearly suffering when using this application method. However, inflorescences of two individuals induced in eppendorf tubes with 17-ß-estradiol showed siliques elongation. Induced plants were screened by using light microscopy 4-5 days of induction. The analysis of these lines revealed seeds containing developing embryos and endosperm. The presence of the developing endosperm suggested that the tested inflorescences were fertilized. One seed containing an abnormal embryo was observed (Figure 3-10). This embryo seemed to grow from integument tissue. The endosperm nuclei in this seed were not observed. However, it may be caused by insufficient clearing. To determine if the seeds development was induced by fertilization event, new inflorescences of lines showing mutant phenotype were induced in eppendorf tubes like before. However, results obtained in the previous experiment were not reproducible. The observed ovules contained only the wild type looking embryo sac without structures resembling developing embryos.

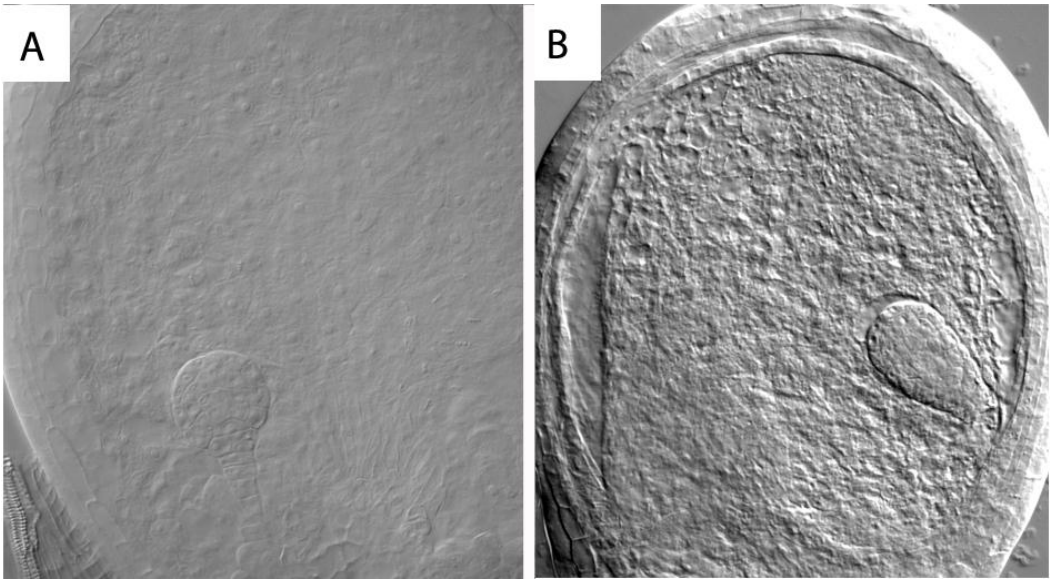


Figure 3-10. Embryos observed in mutant line ectopically mis-expressing *WUS* after induction with 17-ß-estradiol. (A) Normal embryo. (B) Abnormal embryo from the same silique.

3.2.2. Mis-Expression of G1/S Phase Genes in the *Arabidopsis* Egg Apparatus

In addition to the gain-of-function screen, a candidate gene approach was undertaken in order to activate elements of apomixis. We tested ability of candidate genes to induce embryo development prior to fertilization. The selected candidate genes belong to the RBR/E2F/DP pathway controlling the G1/S phase transition of the plant cell cycle. These genes were mis-expressed in the egg apparatus of Arabidopsis male sterile mutant line *dde2-2* of the Columbia ecotype.

The RBR/E2F/DP pathway in plants is controlled by several gene groups. The universal drivers of the G1/S transition are cyclin dependent kinases (CDKs). Their activity is regulated by cyclins (cyc), CDK-activating kinases (CAKs), WEE1 kinases and kip-related proteins (KRPs). CDK-cyclin complexes trigger the G1/S phase transition by the destruction of the transcriptional repressor the RBR/ E2F/ DP complex. The releasing of the E2F and DP transcription factors leads to expression of target genes which are required for the S-phase progression (Gutierrez *et al*., 2002). The more than 20 genes found to regulate this phase of the cell cycle are classified into different groups. The Arabidopsis CDKs belong to six subtypes (A-F). Among all of the CDKs, the only A-type CDK (CDKA;1) seems to be the key player of the G1-to-S phase transition. CDKDs (;1, ;2, ;3) and CDKF function as a CDK-activating kinase (CAKs) and might be involved in the G1/S transition as CAKs were found to phosphorylate CDKA;1. Arabidopsis contains approximately 30 cyclins with putative role in the cell cycle which belong to A- (10 cyclins), B- (11 cyclins), D- (10 cyclins) and H-types (1 cyclin). During the G1 phase cyclins of the D-type, A-type and H-type are expressed. There are eix E2Fs (E2Fa, E2Fb, E2Fc, E2Fd/DEL2, E2Fe/DEL1, E2Ff/DEL3) transcription factors and their binding partners DPs (DPa and DPb) regulate the expression of many genes required for the entry and progression of the S phase in Arabidopsis (reviewed in Rossi and Varotto, 2002; Inzé and De Veylder, 2006; Francis, 2007; Gutierrez 2009).

From all the genes involved in the G1/ S phase progression, eight were selected to be mis-expressed in the Arabidopsis egg cell in order to trigger parthenogenesis (Table 3-1).

Gene	Open Reading Frame Name	Destination Vector
CDKA;1	At3g48750	pMJ2
CDKD;3	At1g18040	pMJ1
CycA3;1	At5g43080	pMJ1
CycD2;1	At2g22490	pMJ1
CycD3;1	At4g34160	pMJ1
CycH;1	At5g27620	pMJ1
E2Fb	At5g22220	pMJ2
DPb	At5g03410	pMJ1

Table 3-1. Table of genes used for the mis-expression experiment. The set of candidate genes consisted of four genes encoding cyclins of A-, D- and H-type, a gene encoding CAK, a gene representing the E2F transcription factor, a gene representing the dimerization partner DP and a CDKA;1.

Candidate genes represented each important subgroup for the G1/S phase transition. We selected these genes based on their expression profiles in the Arabidopsis egg cell. The cell-type-specific transcriptomes were obtained using a combination of the laser-assisted microdissection (LAM) of the individual cell in the Arabidopsis female gametophyte with the Affimetrix ATH1 GeneChip microarray platform (Wuest *et al*., 2010). We were seeking for genes which transctipts were likely absent in the egg cell. According to the data, cyclins, the *E2Fb* transcription factor and the *CDKA;3* were chosen. The *CDKA;1* transcriptome most likely is present but was selected because of its key role in the G1/S progression. No expression data were available for the *DPb* transcription factor.

The coding sequences (*CDS*) of selected candidate genes were amplified from mRNA obtained from leaves of the WT *Arabidopsis thaliana* plant. Using primers with *attB* adapters the *CDS* of selected genes were introduced into the Gateway® cloning cassette of pMJ1 and pMJ2 vectors. The transcriptional expression of candidate genes was restricted to the egg apparatus of the female gametophyte by an egg apparatus specific enhance fused to the CaMV minimal 35S promoter (Yang *et al*., 2005) (Figure 3-11).

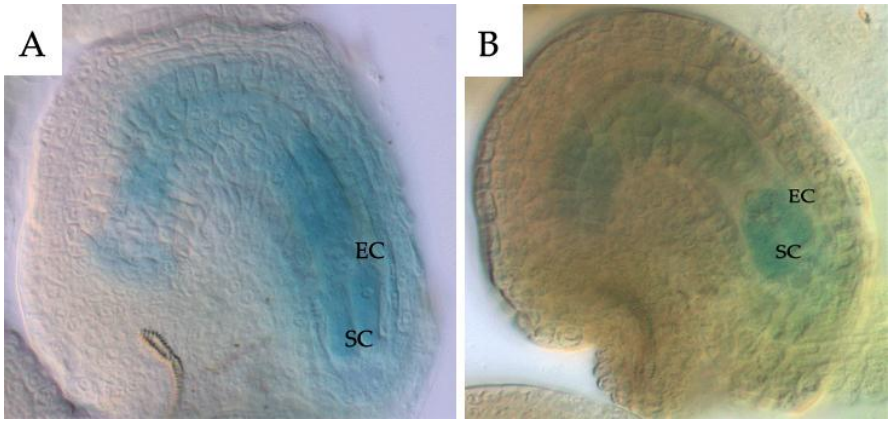


Figure 3-11. Expression pattern of the *GUS* reporter gene in *dde2-2* Col transformed with (A) pMJ1-*GUS* and (B) pMJ2-*GUS* vector. EC- egg cell, SC- synergid cell

The genes encoding the cyclins, CAK, and DPb were inserted into the pMJ1 vector. The genes encoding CDKA;1 and E2Fb were cloned into pMJ2 vector (Table 3-1). Different antibiotic resistance in plants enables selection of mutants containing both for pMJ1 and pMJ2.

The candidate genes were mis-expressed in the male sterile *dde2-2* Col background. The *dde2-2* mutant plants were transformed by the dipping method using *Agrobacterium tumefaciens* GV3101. In total 60 independent T_1 lines were generated. Subsequently, seeds of these lines were selected on MS plates containing the required antibiotic, and the 3: 1 Mendelian segregation ratio of T_2 seedlings was checked in order to estimate the number of T-DNA insertions in the lines generated (Appendix, Table 6-9). Only lines showing 3: 1 segregation were subsequently screened for embryo development in absence of fertilization. Analysis was carried out on carpels of young flowers. Observations by light microscopy were performed on approximately eight individuals per line carrying the T-DNA insertion. Four carpels of each individual mutant plant were dissected and analyzed. In total of 32 carpels per line were screened in order to test if the mis-expression of genes involved in RBR/E2F pathway can trigger the embryo development in the absence of fertilization. Unfortunately, none of the analyzed lines showed parthenogenetic embryo development phenotype. The ovules of these lines analyzed contained wild type looking embryo sacs consisting of synergids, the egg cell and the central cell without structures that hint to the zygote or the embryo development (Figure 3-13)

.

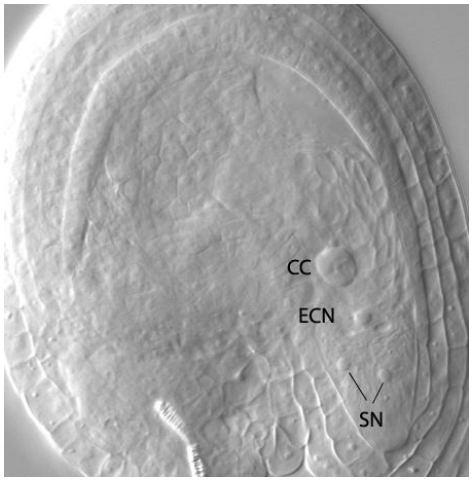


Figure 3-13. Light microscopy image of a WT *Arabidopsis thaliana* ovule containing normal embryo sac. CC- central cell, ECN- egg cell nucleus, SN- synergids nuclei.

The progression of the G1/S phase of the plant cell cycle highly depends on the CDKA;1 which activity is modulated by cyclins or CDK-activating kinases. Such CDK-cyclin complexes trigger the cell cycle progression by the inactivation of the RBR/E2F/DP complex. Thus, crosses between a line overexpressing CDKA;1 and lines over-expressing cyclins and CDKD;3 were performed in order to manipulate the regulatory machinery of the G1/S transition. Using the CDKA;1 mutant as a maternal line, five double mutants were generated by crossing: (i) CDKA;1/CDKD;3, (ii) CDKA;1/CycA3;1, (iii) CDKA;1/CycD2;1, (iv) CDKA;1/CycD3;1 and (v) CDKA;1/CycH;1. The F_1 generation was put on MS plates with required antibiotics to select lines. Carpels of eight double heterozygous mutant plants representing one line were screened using light microscopy. Four carpels per individual were screened for the egg cell activation phenotype. However, no developing embryos could be identified. The tested ovules resembled the wild type phenotype of an unfertilized embryo sac. However, in all lines about 50 % of ovules contained arrested embryo sacs at the stage of the functional megaspore (Figure 3-14).

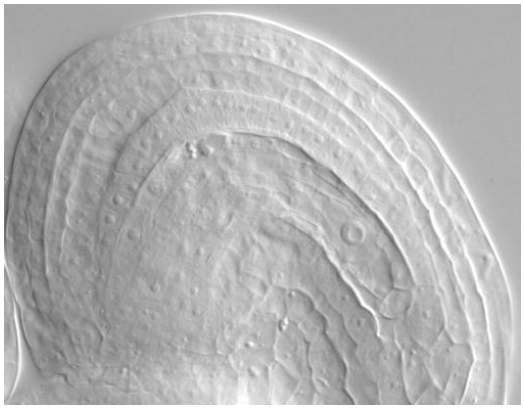
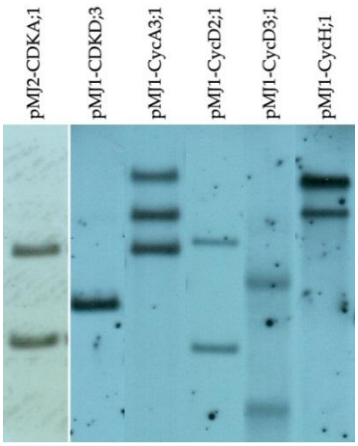


Figure 3- 14. Functional megaspore mother cell observed in double mutants

Southern blot analysis revealed that most lines used for these crosses carried more than one insertion (Figure 3-15A). The only exception was a line with overexpressed *CDKD;3* gene.

New crosses between line overexpressing *CDKA;1* and lines overexpressing cyclins or *CDKD;3* were performed. Lines used for crosses likely carried single insertion (Figure 3-16B). Microscopic observations of the F_1 progeny of these crosses again revealed no developing embryos in embryo sacs. However, no embryo sac abortion was detected in the progeny.

A



B

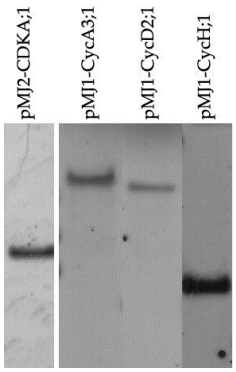


Figure 3-15. Southern blot analysis. (A) Lines used for crosses that revealed multiple T-DNA insertions.(B) Lines used for crosses that contained a single T-DNA insertion.

In the next step, attempts to generate triple or quadruple mutants overexpressing the G1/S phase genes in the egg apparatus were taken in order to induce parthenogenetic embryo development. As all cyclin genes and *CDKD;3* gene were introduced into pMJ1 vector containing kanamycin gene as a plant selection marker, specific primer pairs were designed to allow the genotyping of multiple mutant lines. The primers used for genotyping were designed base on the sequences obtained from Tail-PCR (Appendix Table 6-10). There were two combinations of triple mutants screened at the F_1 generation, *CDKA;1*/+; *CDKD;3*/+; *CycD2;1*/+ and *CDKA;1*/+; *CDKD;3*/+; *CycA3;1*/+ (Appendix, Table 6-11). There were 8 lines of the F_1 generation screened; from each line 4-6 carpels were analyzed for parthenogenetic phenotype, which in case of triple mutants was supposed to be observed in 12.5% of ovules. Microscopic observation revealed that no autonomous divisions of the egg cell resulting in the embryo

development were triggered. It is suggested that during the G1-to-S phase progression, the activity of the CDKA;1 protein is modulated by cyclins D (Boniotti and Gutierrez, 2001). Further, such CDKA;1/cycD complex may be activated by CDKD (Menges *et al*., 2005) and cyclin H (Shimotohno *et al*., 2006) to finally inhibit the RBR/E2F/DP complex leading to the cell cycle progression. Hence, we attempted to crosses the triple *CDKA;1*/+; *CDKD;3*/+; *CycD2;1*/+ mutant line with the line overexpressing *CycH;1* to further manipulate genes of the G1/S phase. However, no quadruple mutants were found in the F_1 progeny. Only triple mutant lines resulting from this cross, such: *CDKA;1*/+; *CDKD;3*/+; *CycH;1*/+ or *CDKA;1*/+; *CycD2;1*/+; *CycH;1/*+ were isolated and screened (Appendix, Table 6-11). Again, light microscopy revealed that there were no developing embryos in the female gametophyte. The *CDKA;1*/+; *CycD2;1*/+; *CycH;1/*+ line, however, contained from ~ 37 % to 53 % arrested at the functional megaspore stage embryo sacs. The *CDKA;1*/+; *CDKD;3*/+; *CycD2;1*/+ line was also used for crosses with quadruple knockout mutant of the genes encoding Kip-Related proteins (*KRP2*, *KRP4*, *KRP6*, *KRP7*) that are known to negatively regulate the G1-to-S transition (De Veylder *et al*., 2001). Due to segregation, in the first generation we were not able to isolate any line that carry overexpressed both *CDKD;3* and *CycD2;1* genes. Two lines heterozygous in all insert loci, carrying either *CDKA;1* and *CycD2;1* or *CDKA;1* and *CDKD;3* in the quadruple heterozygous *krp* knockout mutant background were selected and propagated. The F_2 generation was selected on MS containing antibiotics (kanamycin, basta, sulfadiazine) and genotyped. Because of segregation only three lines were selected for further analysis (Appendix, Table 6-11). One line (13_11) carries overexpressed both *CDKA;1* and *CDKD;3* genes in the homozygous triple *krp* (*krp2*, *krp4*, *krp7*) background. In two carpels, out of ten analyzed, ovules containing structures resembling multiple (double or triple) egg cells were observed (Figure 3-16). However, such embryo sacs were observed at a very low frequency ~ 4.5 %.

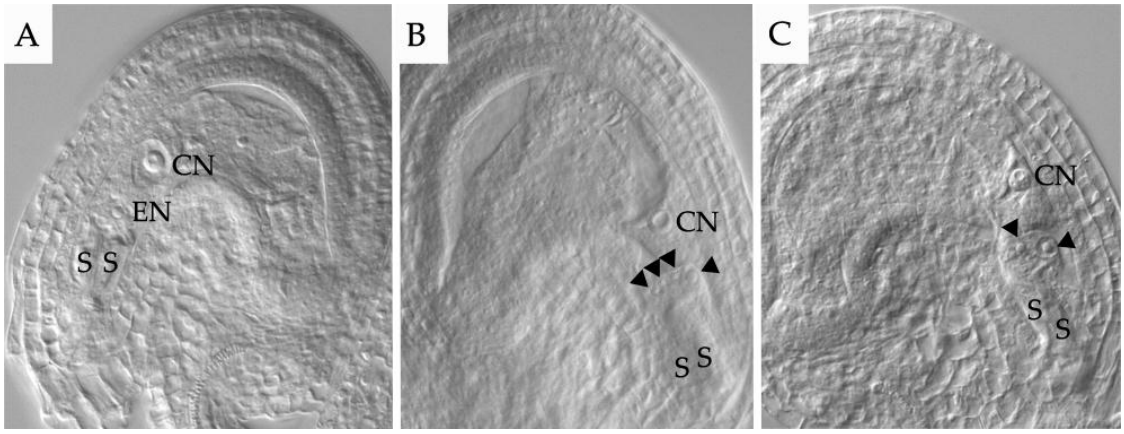


Figure 3-16. Embryo sacs observed in the 13_11 mutant line. (A) WT embryo sac of *Arabidopsis thaliana*. (B) and (C) Embryo sacs containing multiple egg cells. CN- central cell, EN- egg cell nuclei, S- synergid. Arrows indicate multiple nuclei.

Another (13_12) line obatined, overexpressed both *CDKA;1* and *CDKD;3* genes but only *krp2* and *krp6* loci were homozygous, while *krp4* and *krp7* loci were heterozygous. The ovules of this line did not show any abnormalities. One single developing seed that was found contained normal embryo and endosperm which indicated accidental pollination event. The third line (41_24) contained only over-expressed *CycD2;1* and three homozygous *krp* genes (*krp2, krp6* and *krp7*), while the *krp4* locus was found to be heterozygous. Embryo sacs observed in this line showed some abnormalities, such aborted embryo sacs at different stages (Figure 3-17). However, no developing embryos were observed in this line either.

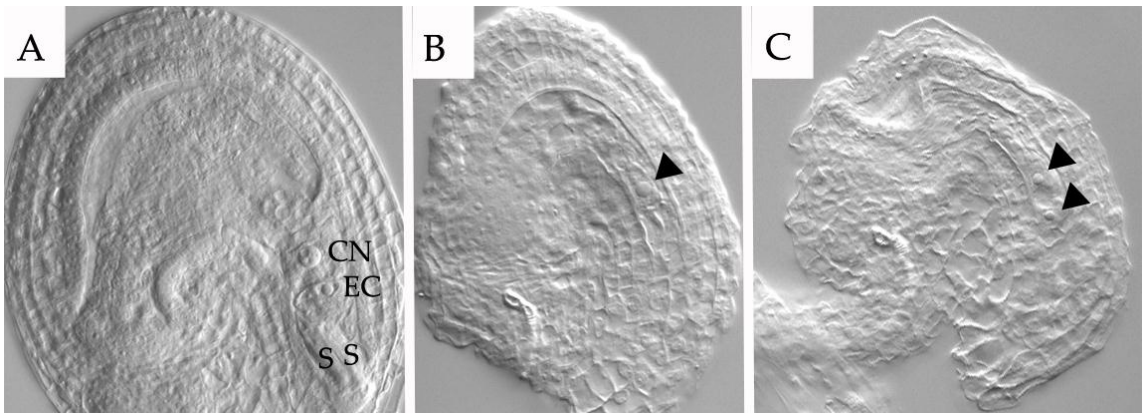


Figure 3-17. (A) Normal *dde2-2* Col embryo sac. (B) Embryo sac aborted at functional megaspore mother cell stage. (B) Embryo aborted at two nucleate stage. Arrows indicate nuclei.

3.2.3. RBR1 and MSI1 Gene Silencing Using Artificial MicroRNAs

In order to silence the *MSI1* and *RBR1* genes we chose an artificial microRNA-based gene-silencing approach (amiRNA). Artificial microRNAs to target these genes were generated using the amiRNA design algorithm using the WMD3 Web MicroRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi; Schwab *et al*., 2006; Ossowski *et al*., 2008). The resulting amiRNA sequences were introduced into the expression vector pMJ1 using the Gateway™ cloning technology. The transcription of the amiRNAs against *MSI1* and *RBR1* was restricted to the *Arabidopsis thaliana* egg apparatus by using an egg apparatus specific enhancer (Yang *et al*., 2005). Similar to previous experiments the conditional male sterile *dde2-2* line was. We obtained eight T_1 lines overexpressing amiRNA targeting *MSI1*. The ovules of these lines were screened using light microscopy. One line exhibited a silique elongation phenotype and produced seeds. Seeds of this line contained normal embryos and endosperm. These observations and presences of pollen suggest a wild type origin of this line. Screening of the remaining lines did not reveal any structures resembling a developing embryo. The unfertilized ovules of this lines contained wild type Arabidopsis embryo sacs without abnormalities. Although selection of transformats was successful and a sufficient number of T_1 seedlings carrying amiRNA against *RBR1* transcripts were obtained, seedlings of these lines died after they were transfer from plates containing selection media to soil.

4. DISCUSSION

4.1. Overview

A rising world population, global climate change, urbanization and land destruction result in growing demands on agriculture. Currently, more than 1 billion people suffer from chronic malnutrition which is particularly harmful for woman and children. Therefore, it is very important to ensure an access for all people to food which meets their dietary needs. It is predicted that the agricultural yields should increase at least 70 % by 2050 to ensure food security for the growing world population (FAO data). Taken together, there is an urgent need to develop new, integrated approaches resulting in the higher production per unit area and a better adaptation of crops to changing climatic conditions. At the same time, there are demands that food should be produced in an environmentally friendly manner. These goals can be achieved by combining traditional plant breeding strategies with novel genetic, molecular and cytological methods. The combination of these methods will allow generating new varieties and traits that are better adapted to the different climatic regions of the world. One great breakthrough in agricultural research could be to employ of apomixis in plant breeding.

Apomixis is an asexual mode of plant reproduction through seeds and it results in a generation of natural clones (Nogler 1984a). The offspring of the apomictic plant are produced parthenogenetically from an unreduced embryo sac and they are an exact genetic copy of the mother. This feature of apomixis could revolutionize agriculture by greatly facilitating the maintance and the propagation of heterosis (Spillane *et al*., 2005). Heterosis or hybrid vigor is a result of crossing genetically distant lines. The progeny obtained from such crosses commonly show an increased performance (yield or other agronomical trait) when compared with their respective parents, as for example illustrated in fox millet in which the yield of a hybrid population was 68 % greater than the average yield of the parental cultivars (Siles *et al*., 2004).

Apomixis is widely distributed among Angiosperms, but its prevalence in agronomical important crops is rather poor. There have been several breeding programs developed in order to introgress the elements of the asexual reproduction from wild apomictic relatives into valuable food crops through hybridization. For example, maize was crossed with its apomictic relative Tripsacum or pearl millet with Pennisetum. These attempts, however, failed due to numerous difficulties, such as loss of apomixis during introgression or failure to set seeds (Savidan, 2001; Grimanelli *et al*., 2001; Spillane *et al*., 2001). The incorporation of apomixis into plant breeding programs demands better understanding of sexual and apomictic processes. Genetic mechanisms controlling the apomixis development still remain unclear and may be as diverse as the apomictic species producing them. Attempts to identify genes controlling apomixis by mapping the trait in the progeny of the apomictic species with sexual relatives showed that apomixis is inherited in a Mendelian manner as one (Savidan, 2000) or more dominant traits (Albertini *et al*., 2004). Unfortunately, the resolution of a mapping approach was insufficient suggesting that apomixis loci are located in non-recombining genomic regions. Thus, breeding and molecular mapping of apomixis components have not revealed the mechanisms that confer apomixis. In this thesis we focused on alternative approaches using *Arabidopsis thaliana*, with its well characterized genetic tools, to identify genes that could be used to engineer the elements of apomixis *de novo.*

The aim of this thesis was identification of genes which are able to trigger elements of apomixis in the female gametophyte of *Arabidopsis thaliana*. As a first approach, relying on the theory that apomixis displayes a deregulation of the sexual pathway (Grossniklaus *et al*., 1998 Grimanelli *et al*., 2001; Koltunow and Grossniklaus, 2003); we performed an activation tagging screen to randomly activate genes in the egg apparatus to initiate elements of parthenogenesis. The inducible two-component system allowing spatial and temporal control of gene expression was used in this experiment (Brand *et al*., 2006). This system relies on the XVE chimeric transcription factor. Upon induction with 17-*ß*-estradiol, XVE transactivates genes adjacent to its target binding sequence *OlexA-TATA* (Zuo *et al*., 2000). The gain-of-function screen was performed in conditional male sterile *Arabidopsis thaliana* mutant *dde2-2* background. This mutant line facilitated screening for silique elongation in absence of fertilization. However, during the screen we did not identify mutants exhibiting elements of parthenogenetic development.

As a parallel approach, we tested several candidate genes for their ability to trigger the parthenogenetic embryo development upon mis-expression in the egg apparatus. Preliminary data obtained by our group indicated *WUSCHEL* as a potential candidate for mis-expression experiments. Inducible ubiquitous expression of this gene in floral tissues showed the viable seed development prior to fertilization (L. Brand, PhD thesis, 2007). We performed further experiments in order to support a hypothesis that *WUS* can trigger the embryo development as on roots. Experiments carried out using male sterile mutants in Col as well as in L*er* background could not confirm a preliminary finding.

To overcome the suspected cell cycle arrest in the egg cell prior to fertilization (Sundaresan and Aledante-Saez, 2010), genes positively regulating the G1-to-S phase progression of the plant cell cycle were used for mis-expression experimants in the egg apparatus. Products of these genes are involved in a suppression of the RBR1 protein which is known as an inhibitor of the G1/S progression (Ebel *et al*., 2004). It was suggested that RBR1 may be required for maintaining of the Arabidopsis egg cell in the G1 phase (Sundaresan and Alandete-Saez, 2010). We selected eight genes important for the RBR1 inactivation to estimate their ability to induce embryo development in absence of fertilization. However, the over-expression of these genes in the Arabidopsis egg apparatus did not result in the RBR1 inactivation that may led to the egg cell activation.

Additionally, we took a gene silencing approach in order to trigger the parthenogenetic embryo development. It was also shown that mutation in the *MULTICOPY SUPPRESSOR OF IRA 1* (*MSI1*) gene leads to a formation of the embryo without fertilization. However, developing embryo aborts at early stages (Guiton *et al*., 2004). The MSI1, similarly as RBR1, may prevent the egg cell activation prior to fertilization. Base on this assumption, we designed artificial micro RNA constructs to silence the expression of both *MSI1* and *RBR1* in the Arabidopsis egg apparatus in order to induce embryo development without fertilization. Also in this case there were no developing embryos observed in the Arabidopsis female gametophyte.

4.1.1. The Gain-of-Function Screening Approach to Induce Parthenogenetic Embryo Development

The common features of apomictic and sexual reproduction modes show a strong evolutionary relationship which suggests that apomixis results from the deregulation of the sexual process in time and space (Grossniklaus, 2001; Grossniklaus and Koltunow, 2003). It means that initiation of elements of apomixis takes place at the wrong time and in the wrong cell type, the apomictic embryo sac development initiates prior to meiosis and embryo is formed prior to fertilization. Depending on where and when this deregulation occurs, different types of apomixis can be produced. Furthermore, elements of apomixis are known to be regulated by a dominant, Mendelian trait. Genetic studies revealed that in some species apomeiosis and parthenogenesis co-segregate (Savidan, 2000), while in others they can segregate independently (Sokolov, 2000; Albertini *et al.*, 2004). These traits, which may appear to be complex, are dominant to the wild type. Hence, a gain-of-function strategy was considered to be a promising approach to identify genes controlling parthenogenesis as key element of apomixis.

To deregulate the expression of genes involved in the plant sexual reproduction the two component system allowing tissue-specific gene induction in Arabidopsis was used (Brand *et al*., 2006). This system provides reliable and versatile conditional gene activation in restricted tissue or cell types. It takes advantage of two components: (i) an activator T-DNA unit and (ii) a responder T-DNA unit. The activator unit contains XVE (Zuo *et al*., 2000), a chimeric transcription factor, which was composed by a fusion of the DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E). The VP16 activation domain, an element of the chimeric transcription factor XVE is known to be a strong activator capable of long-range transcriptional activation (Laybourn and Kadonaga, 1992). It stimulates transcription by targeting histone acetylating complexes to the nucleosomal template causing a large-scale heterochromatin de-condensation (Tumbar *et al*., 1999; Vignali *et al*., 2000).

The transcription of the XVE activator in this system is controlled by cell-type specific cis-elements. Upon activation with chemical inducer – estradiol – XVE binds to its responsive promoter *lexA* which leads to the transactivation of adjacent genes. The two

component inducible system was shown to faithfully induce genes in the cell-specific manner (Brand *et al*., 2006). Generation of conditional gain-of-function mutants (*35S*, *SUC2*, *GL2* or *TobRB7*) in non-reproductive tissues showed the universal application of this method. In more than 72 % of the transformants the expression pattern of the target gene was reliably expressed in these tissue types, regardless of the position of the activator or responder units in the plant genome. Experiments with the diphtheria toxin A-chain (*DT-A*) cytotoxin gene, which inhibits protein synthesis, demonstrated the stringency of the system. Mutant plants with the responder unit containing *DT-A* grown in the absence of estradiol showed no phenotypic effects. Thus, this system allows tight regulation of theexpression of the gene of interest (Brand *et al*., 2006). It was also shown that using the XVE system it is possible to modulate the expression of genes by applying different concentrations of the inducer (Zuo *et al*., 2002).

In order to limit the expression of the chimeric transcription factor XVE to the egg apparatus, an Arabidopsis egg apparatus specific enhancer (*AtEASE*) (Yang *et al*., 2005) was employed. The *AtEASE* was found using enhancer detection approach and it was shown to be highly efficient in controlling expression of genes specific for the egg apparatus (an egg cell and synergids). The EASE itself in not functional, however, when fused with CaMV 35S minimal promoter can drive the expression of genes, restricting their activity to nuclei of the egg apparatus starting from cellularization until late stages of the female gametophyte development and after fertilization in the zygote until the globular embryo stage (Yang *et al*., 2005). Thus, the expression pattern of the egg apparatus specific enhancer suggest it potential use in engineering of apomixis elements. Since the *AtEASE* sequence shows no activity at other stages of plant development it is ideal to control the mis-expression of genes inducing embryo development

The two-component system was successfully used to conditionally activate reporter gene expression (*GUS*) in the egg apparatus of Arabidopsis in plants transformed with pLB12-*AtEASE* (Brand *et al*., 2006). Using the *AtEASE* enhancer it is possible to restrict the expression of XVE to the egg apparatus and transactivate T-DNA tagged genes in these cell-types, only in the presence of the chemical inducer – estradiol. In apomictic species, the initiation of the embryo development in the absence of fertilization most likely is related to the mis-expression of genes in the egg cell. Therefore, the egg cell is a good cell-type to ectopically express genes in order to induce parthenogenesis. This hypothesis could be supported by studies performed in the “Salmon” wheat line. In vitro

experiments showed that unfertilized egg cells are able to develop into an early embryo without fertilization and in the absence of ovary-derived signals (Kumlehn *et al*., 2001) suggesting that egg cell activation can be independent from external factors. However, the molecular mechanisms of this phenomenon are not known. Moreover, in many Angiosperm species, parthenogenesis is described to occur spontaneously at low frequency (Kimber and Riley, 1963). Also the production of haploid plants was observed in maize (Sarkar and Coe, 1966; Kermicle 1969) or barley (Hagberg and Hagberg, 1980). Morphological observation of haploids suggests that such individuals are weaker in comparison to diploids counterparts (Jauhar, 2006); however, they can achieve maturity or even produce seeds (Ravi and Chan, 2010). Spontaneously formed haploids were also observed in durum wheat and displayed reduced height, narrow leaves and a thinner steam when compared to the diploid parental cultivar. Similar observations were made in haploid *Arabidopsis thaliana* (Ravi and Chan, 2010).

In order to avoid hand emasculation and an accidental pollination leading to generation of false positive lines, the tightly regulated conditional male sterile *delayed dehiscence 2-2* (*dde2-2*) mutant line (von Malek *et al*., 2002) in the Columbia background was used for the purpose of this screen. Phenotype of this mutant line is caused by a null mutation in the *ALLENE OXYDE SYNTHASE* (AOS), gene encoding an enzyme important for jasmonic acid biosynthesis. The null mutation results from a transposon after En1/Spm1 transposon-induced mutagenesis (Wisman *et al*., 1998). The *dde2-2* mutant can be rescued by the application of methyl jasmonate by spraying the inflorescences. The mutation has no effect on the female gametophyte and upon fertilization with wild type pollen this plant line displays normal seed set. Silique elongation in a male sterile mutant may suggest the apomictic seed development, and such an easy to score phenotype is crucial in a screen. Siliques will elongate even when only a small number of cell division takes place in the developing seed (Ohad *et al*., 1996; Chaudhury *et al*., 1997; Kiyosue *et al*., 1999). For the purpose of our screen the *dde2-2* mutant line was used to generate the activator line (LB122) containing the chimeric transcription factor XVE under the transcriptional control of the *AtEASE* promoter. The LB122 line shows inducible expression of the reporter gene *GUS* that is restricted to the egg apparatus. This allows screening for conditional gain-of-function mutants displaying silique elongation independently from fertilization. The activator line was super-transformed with the responder vector for the gene tagging. T-DNA transformants obtained are

normally hemizygous for the T-DNA insertion (Ye *et al*., 1999), therefore T_2 and later T_1 plants were screened for the dominant inducible phenotype.

It was calculated that all transformants will generate an inducible activation-tagged gene. The Arabidopsis genome size is approximately 125 Mb and consists of ~ 25 000 genes, with an average length of 2,013 kb and average density of ~ 1 gene per 4.2 kb (The Arabidopsis Genome Initiative, 2000) thus ~ 47 000 transformants should be required in order to be 95% confident of inserting at least one tagging T-DNA in any given 8 kb region of the Arabidopsis genome (from $P= 1- (1- [x/ 125\ 000])^n$, where P= 0.95, x= distance [kb], n= number of required transformants) (Krysan *et al.*, 1999). Since the XVE chimeric transcription factor is capable to facilitate the activation of genes up to 8 kb from the DNA binding site (Zuo *et al*., 2002; Sun *et al.*, 2003) it should be possible that each insertion is able to activate a gene in the Arabidopsis genome. However, a screen on such scale would be impractical. This project aimed in identification of a few candidate genes that might be important for apomictic development. Therefore, the goal of this screen was to look at approximately 10000 transformants, sampling about one third of Arabidopsis genes.

During this screen approximately 9000 transformant lines were screened for silique elongation in absence of fertilization. The T_2 and later T_1 plant generations were screened for a dominant inducible phenotype. The decision to change the screening population form T_2 to T_1 was made in order to reduce time and work force resources. Furthermore, we found that the responder unit isolated from GV3101 competent cell and form super-transformants most probably underwent recombination during bacteria cells propagation which resulted in shortening of the lexA. Therefore, the new tagging construct containing shorter and more compact insertion fragment was generated. But such recombination was observed also in case of the new tagging vector to some degree. The performed screen did not reveal any parthenogenetic embryo phenotype. None of potential candidates selected during the screen show the embryo development in the ovules from carpels under estradiol induction. The occasional silique elongation and the seed development observed in a few potential candidates were proven to be result of accidental pollination or seed contamination and did not display induced parthenogenesis. Moreover, in no case we were able to confirm the phenotype in the next generation.

The genetic basis of parthenogenesis is poorly understood. Data obtained from experiments on genetic control of apomixis are equivocal. Early studies suggested that parthenogenesis is closely linked to apospory in Ranunculus (Nogler, 1984b) and completely linked to apospory in Panicum (Savidan, 1980). These findings resulted in hypothesis that the expression of parthenogenesis is presumably a result of the pleiotropic effect of the apospory or the diplospory (Nogler, 1984b). On the other hand, recent data indicated an independent inheritance of parthenogenesis and apomeiosis (Noyes and Riesberg, 2000; van Dijk *et al*., 2004; Catanach *et al*., 2006, Kaushal *et al*., 2008). It is not possible to exclude that the activation of such complex programs like apomeiosis or parthenogenesis requires more factors (Grimanelli *et al*., 2001). These factors could act upstream and regulate the activity of genes which mutations lead to development of elements of apomixis (Grossniklaus *et al*., 1998; Luo *et al*., 1999; Ohad *et al*., 1999).

The data obtained in *Poa pratensis* support a multigenic model of the parthenogenesis development (Matzk *et al.,* 2005). In this species two genes, out of five identified to be required for the apomictic reproduction, control parthenogenesis: the *Parthenogenesis initiator* (*Pit*) and the *Parthenogenesis preventer* (*Ppv*). Also studies of the apomeiotic locus revealed that the structure of the *DIPLOSPOROUS* (*DIP*) locus in *Taraxacum officinale* may consists of minimum two *DIP*-genes (Vijverberg *et al*., 2010) controlling this element of apomixis. Further studies performed in *Erigeron annuus* (Noyes, 2005) also support a multigenic control of apomeiosis.

The attempt to induce parthenogenesis in sexual *Arabidopsis thaliana* undertaken during this PhD studies did not result in the isolation of a parthenogenetic mutant. As the onset of parthenogenesis could depend on multiple factors it might be difficult to activate all that are required to trigger parthenogenetic development of the embryo. A possibility is that we overlook very early aborted stages of the embryo development when looking for silique elongation phenotype.

The diploid male sterile *Arabidospsis thaliana* mutant was used to perform the activation tagging screen in attempt to induce parthenogenesis. However, different hypothesis associating the apomictic development with polyploidy were established since almost all gametophytic apomicts are polyploids (van Dijk, 2009). According to these theories, an expression of apomixis is dosage dependent. That means that diploid

plants may carry apomixis genes, however, they occur below a required dosage ratio and are not expressed (Mogie, 1992, discussed in Noyers and Riesberg, 2000). It was also suggested that apomixis genes are dominant but have a recessive lethal effect which is exhibited when they occur in the haploid male gamete in the absence of compensatory wild type alleles (Nogler, 1984b).

In apomictic *Erigeron annuus*, parthenogenetic seed production was only observed in plants that are diplosporous. Although markers linked to a parthenogenesis locus occurred in meiotic plants, parthenogenetic seeds were never observed. This was true for sexual diploid and sexual triploid hybrids. This finding suggested that the parthenogenetic embryos can develop from the reduced egg cell but abort prior to the seed formation. Alternatively, the absence of parthenogenesis in sexual plants is because the expression of the trait is contingent upon a diplosporous background (Noyes and Riesberg, 2000). Further, data indicated that the autonomous embryo and the endosperm development can occur in diplosporous as well as meiotic individuals (Noyes *et al*., 2007). However, seeds from meiotic plants underwent abortion and failed to achieve maturity. This finding refutes the previous theory that the parthenogenesis in Erigeron is only expressed in the diplosporous background. However, it still suggests that an unreduced embryo sac is required for successful parthenogenetic seed (Noyes, 2006). Thus, we may conclude that a haploid embryo sac and a low a ploidy level may reduce the frequency of the autonomous seed development.

The apomeiotic mutant in the male sterile background with still optimized induction protocol and subsequently screening by light microscopy would be points to improve in this convincing gain-of-function approach.

4.2. Candidate Gene Approach

4.2.1. WUS

The cloning of candidate genes that may induce apomixis is one of the strategies which can be applied to introduce this trait into crop species (Spilane *et al*., 2004). Genes with a proven role in the sexual reproduction are good candidates for mis-expression experiments in order to induce elements of parthenogenesis. This group of

potential candidates includes genes whose mis-expression leads to the somatic embryo development on different plant tissues, such as *BBM* (Boulilier *et al*., 2002), *WUSCHEL* (Zuo *et al*., 2002), *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (Schmidt *et al*., 1997; Hecht *et al*., 2001), *LEC1* (Lotan *et al*., 1998) or *LEC2* (Stone *et al*., 2001). Previous experiments carried out in our lab (Brand, PhD thesis, 2007) suggested that *WUSCHEL* (*WUS*) might induce embryo development when mis-expressed in the female gametophyte prior to fertilization. In Arabidopsis the *WUS* gene regulates stem cell fate during the development. Mutations in *WUS* lead to failure in self-maintenance of the shoot and floral meristems (Laux *et al*., 1996). The expression of this gene is detected in the group of cells underlying the stem cell of the shoot meristem preventing their differentiation (Mayer *et al*., 1998). In addition to the role in shoot and floral meristems, *WUS* was found to be expressed in the nucellus and is involved in coordination of the ovule integument formation from the chalaza (Gross-Hardt *et al*., 2002). Ectopic overexpression of *WUS* promotes the vegetative-to-embryonic transition and results in the somatic embryo formation on diverse plant tissues, most efficiently on roots suggesting its crucial role during embryogenesis (Zuo *et al*., 2002). Further, mutants ectopically expressing *WUS* produce ectopic meristems (Xu *et al*., 2005). The type of the meristem, however, is depended on developmental and physiological stages of the plant, e.g. floral meristems were preferentially formed on inflorescences, whereas shoot meristems producing leaf like structures on leaves. Hence, *WUS* overexpression in the female gametophyte could potentially promote the development of the embryo.

In previous experiments *WUS* was mis-expressed using ubiquitously active synthetic promoter *GT10-90* (L. Brand, PhD thesis, 2007). Plant lines induced with 17-*ß*-estradiol produce elongated siliques containing seeds. Observations of seeds revealed broad range of embryo development from early heart stages to mature embryos. Furthermore, in ovules containing early heart stage embryo endosperm was not observed. The results obtained from these experiments suggested that ubiquitous expression of *WUS* might lead to the embryo development without fertilization. However, restriction of *WUS* expression to the egg apparatus was insufficient to induce the embryo formation which suggests that, the embryo originates from other cell than the egg cell or may require a diploid egg cell (L. Brand, PhD thesis, 2007). Nevertheless, these promising results required further experiments confirming these findings, mainly because the mis-expression of *WUS* was carried out using *Arabidopsis thaliana* WT plants and it may be

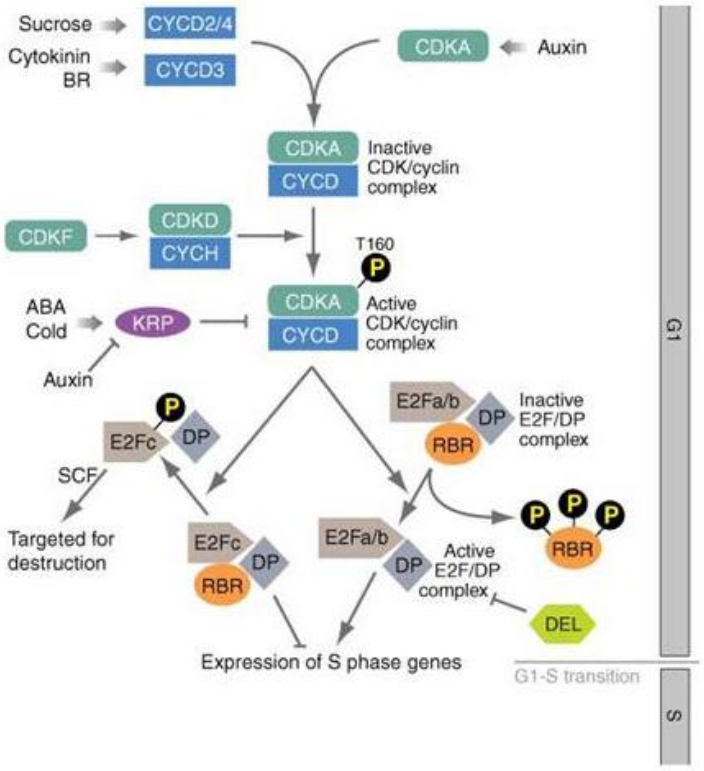
possible that observed embryos were the result of pollination. The Arabidopsis conditional male sterile mutant line *dde2-2* in Columbia background was used to continue these experiments to exclude need of emasculation and reduce a risk of accidental pollination. This line was super-transformed with an inducible *WUS* construct (pMDC113). Unfortunately, no developing embryos were observed in the female gametophyte after induction with 17-*ß*-estradiol. We assumed that embryo formation prior to fertilization resulting from *WUS* mis-expression may also depend on the Arabidopsis ecotype background. Therefore, the conditional male sterile line *dde2* of the Landsberg *erecta* ecotype was also super-transformed with the inducible *WUS* construct. However, induction with estradiol did not result in a parthenogenetic phenotype. Although the ectopic expression of genes, such as *WUS* or those mentioned above, stimulates somatic embryogenesis in culture or on vegetative organs, there is so far no evidence that they would induce parthenogenesis within a seed, which is requirement for apomixis (Spielman *et al*., 2003). The mis-expression of *AtSERK1* in the female gametophyte in *Arabidopsis thaliana* did not result in potential apomictic either (Kantama, 2005).

4.2.2. Mis-Expression of the G1-to-S Transition Genes

In addition to genes regulating the sexual pathway, other genes could be used to induce elements of apomixis in non-apomictic species. This group might include genes involved in callose degradation, cell wall formation or genes important for cell cycle progression (Grossniklaus, 2001). Since the Arabidopsis egg cell is proposed to be arrested at G1/ S (Sundaresan and Alandete-Saez, 2010), we decided to mis-express genes necessary for the transition of this phase in an attempt to activate the egg cell to develop into an embryo. Candidate genes were overexpressed in the egg apparatus of the conditional male sterile mutant *dde2-2* (von Malek *et al*., 2002) under the transcriptional control of the *AtEASE*/min35S promoter (Yang *et al*., 2005).

The cell cycle is a tightly regulated process resulting in the production of two daughter cells identical to their mother. The progression through the entire cell cycle encompasses several alternating events. First, after completing of the previous division, the post-mitotic interphase (G1) takes place, then the DNA synthesis phase (S-phase), the post-synthetic phase (G2) and finally mitosis (M) occur. At the G1 phase, a cell either re-

enters the cell cycle by passing throughout the restriction point or exits from the cell cycle temporally or permanently (Murray *et al*., 2001). The transition from the G1 to S phase is controlled by the highly conserved the RB/E2F/DP pathway (Harbour *et al*., 2000; Ramirez-Parra and Gutierrez, 2000) (Figure 4-1). The Retinoblastoma protein (RB) interacts with the E2F/DP complex, preventing the progression to the S phase. The E2F family of transcription factors and their dimerization partners DPs regulate the expression of genes required for the entry into the S phase and the cell cycle progression (Ramirez-Parra *et al*., 2003). The Arabidopsis genome contains six E2Fs: E2Fa, E2Fb, E2Fc, E2Fd/DEL2, E2Fe/DEL1, E2Ff/DEL3 and two DPs: DPa and DPb (De Veylder *et al*., 2002; Magyar *et al*., 2000; Vandepoele *et al*., 2002).



Inzé and de Veyler, 2006

Figure 4-1. Regulation of the G1/S phase transition. In the presence of growth factors (sucrose, auxin, cytokinon, brassinosteroids) cyclins D (CYCD) from an inactive complex with CDKA;1. This complex is probably activated by the CDK-activating kinase pathway, which involves CDKF and CDKD associated with a cyclin H (CYCH). The activated CDK/CYCD complex is negatively regulated by KRPs. The CDKA/CYCD complex triggers the G1-to-S by the destruction of the E2Fc/DP/RBR transcription repressor complex and by releasing the transcriptional activity of the E2Fa-b/DP/ RBR complexes. As a result, the expression of S-phase genes is activated. The DEL transcription factors might fine-tune the expression of the E2F target genes.

The E2F/DP complexes are inactive when the RB protein binds to it. The activity of these complexes is restored upon phosphorylation of the RB by the CDKs/cyclin complex. The Arabidopsis genome contains only one Retinoblastoma related protein (RBR1) (Durfee *et al*., 2000) that was also found to function as a negative regulator of the cell proliferation during megagametogenesis (Ebel *et al*., 2004). It was shown that Arabidopsis knock-out mutants of the *RBR1* gene are gametophytic lethal because they fail to arrest mitotic divisions in the mature female megagametophyte which results in excessive nuclear divisions in the embryo sac. In these mutants autonomous endosperm development was also observed. Thus, there is an indication that Arabidopsis RBR1 is involved in the arrest of the mature, unfertilized embryo sac.

During the G1/S phase transition different genes, such as cyclin-dependent kinases (CDKs) and cyclins are expressed (reviewed in Rossi and Varotto, 2002; Gutierrez *et al*., 2002; Francis, 2007). Based on the sequence comparison with eukaryote homologues, different plant CDKs were identified and classified into five subgroups (A – E) (Joubes *et al*., 2000). The A-type CDK (CDKA;1) is known to be the universal driver of the G1/ S phase progression in plants. It was shown that the overproduction of the dominant negative *CDKA* of *Arabidopsis thaliana* in *Nicotiana tabacum* plants led to the overall reduction of the cell division rate (Hamerly *et al*., 1995). The activity of the CDKA;1 protein is positively modulated by cyclins (cyc). In the *Arabidopsis thaliana* genome 32 cyclins with a putative role in the cell cycle progression were identified and further divided into for subgroups (10 A-type, 11-B-type, 10 D-type, 1 H-type) (Vandepoele *et al*., 2002). In a broad sense, cyclins belonging to the D-type, A-type and H-type are thought to regulate the G1-to-S transition (Boniotti and Gutierrez, 2001; reviewed in Inze and De Veylder, 2006; reviewed in Francis, 2007; Takahashi *et al*., 2010). Several experiments studying overexpression effects of the G1/S phase genes, such as: *CycD2;1* (Cockcroft *et al*., 2000; Qi and John, 2007) and *CycD3;1* (Menges *et al*., 2006; Schnittger *et al*., 2002) revealed the ability of these genes to stimulate the progression through the G1 phase and promote the S-phase entry and mitosis, respectively. Further, the CDKs/ cyclin complexes are known to be positively regulated by CDKs which are classified as cyclin-dependent kinase activating kinases (CAKs). Arabidopsis contains four genes encoding CAKs which belong to two functional classes: CDKD (CDKD;1 ;2 ;3) and CDKF (Umeda *et al*., 1998; Vandepoele *et al*., 2002;

Umeda *et al*., 2005). In the negative regulation of the cell cycle progression, cyclin-dependent kinase inhibitors (CKIs) are involved (De Veylder *et al*., 2001). There are seven CKI proteins, Kip-Related Protein 1 (KRP1) to Kip-Related Protein 7 (KRP7) described for Arabidopsis, each of different structure and CDK binding specificity.

A selection of candidate genes based on the Arabidopsis egg-specific expression profiling (Wuest *et al*., 2010). We identified genes which transcripts were likely not expressed in the egg cell. Lack of transcripts of these genes may suggest that their absence prevents the egg cell from further divisions before fertilization. Furthermore, the selected group of candidates was supposed to comprise representatives of all gene subclasses involved in the G1/S transition. Based on these assumptions we selected eight genes: *CDKA;1*, *CDKD;3*, *CycA3;1*, *CycD2;1*, *CycD3;1*, *CycH;1*, *E2Fa* and *DPb* for the overexpression in the egg apparatus. Although, the *CDKA;1* transcript was present, we decided to use this gene in the experiment because it is a key component of the G1/S phase progression. The *DPb* gene was mis-express because it is one of the two E2F dimerization proteins acting as positive regulators of the G1/S phase.

Microscopic observations of the obtained single transformants revealed that overexpression of single genes involved in the G1/S phase transition did not induce divisions of the egg cell. In a next step, we tested weather double transformants overexpressing *CDKA;1* and one of cyclins are able to trigger the parthenogenetic embryo development. It is known that the activity of CDKs depends on a noncatalytic partner, the cyclin. In *Arabidopsis thaliana* both D2 and D3 (Boniotti and Gutierrez, 2001; Healy *et al*., 2001) as well as A3;1 (Takahashi *et al*., 2010) cyclins were shown to regulate the activity of CDKA;1 leading to an inactivation of the RBR protein (Boniotti and Gutierrez, 2001; Healy *et al*., 2001). The CDKD;3 protein was found to be involved in the activation of CDKA;1/cyclin complexes before they can phosphorylate RBR (Umeda *et al*., 2005). It may also play a role in the activation of the CDK activity during a re-entry of the cell cycle (Menges *et al*., 2005). Further, in the CDKDs positive regulation CycH;1 may be involved (Shimotohno *et al*., 2006). Considering these interactions between different cell cycle proteins, we preformed crosses between lines overexpressing the *CDKA;1* gene with lines overexpressing *CycA3;1*, *CycD2;1* and *CycD3;1* genes. Additionally, the line overexpressing *CDKA;1* was used to perform crosses with plant lines overexpressing *CDKD;3* and *CycH;1*. Similarly to single transformants, observation of lines overexpressing both components of the

CDKA;1/cyclin complex or CDKA;1/CDKD;3 carried out by using light microscopy did not reveal an expected phenotype, such as a zygote development in absence to fertilization. In the progeny of these crosses, we observed ovules carrying embryo sacs arrested at the functional megaspore stage. It was problematic to associate such phenotype with the activity of the *AtEASE/*min35S promoter used in this study to overexpress candidate genes. It was demonstrated that the activity of this promoter begins at a four-nucleate stage that is after the functional megaspore phase (Yang *et al*. 2005). However, such unlinked gametophytic lethal events can be explained by translocations and other rearrangements of maternal chromosomes during the integration of the T-DNA. It was shown that among the T-DNA insertion lines, a rather high percentage (8 %) of lines exhibited a female semisterile phenotype that was not due to the insertion but it was caused by rearrangements and translocations of chromosomes during mutagenesis (Johnston *et al*., 2007). Moreover, crosses of the line overexpressing the *CDKA;1* gene with lines overexpressing cyclins were repeated using different mutant lines than in the previous attempt. In the progeny of the repeated crosses we did not observe arrested embryo sacs. This observation emphasizes a presumption that embryo sac lethality resulted from the chromosomal translocations and rearrangements during mutagenesis rather than promoter activity. Further, to mimic putative interactions between components of the G1/S transition, crosses were performed to obtain multiple mutants overexpressing genes driving this process. However, also observations of triple mutants overexpressing genes promoting the G1-to-S transition carried out by the light microscopy did not reveal any phenotype suggesting the cell cycle progression.

We hypothesized that attempts to overexpress selected candidate genes may not lead to the RBR1 phosphorylation resulting in the cell cycle progression and divisions of the egg cell. We did not analyze levels of the candidate gene transcripts in obtained mutant lines. Thus, we do not know if transcripts of these genes were elevated when overexpressed in the egg apparatus in comparison to the wild type Arabidopsis plant. However, the overexpression of the *GUS* reporter gene using pMJ1 and pMJ2 vectors resulted in blue staining in the egg apparatus suggesting that these vectors are useful for the overexpression experiment.

To mis-express candidate genes we inserted their cDNA sequences into the Gateway cloning cassette of expression vectors. It was reported in plants, that cDNA transgenes seem to be at risk of the incomplete expression resulting in truncations of the gene

transcript (Brendel *et al*., 1998). Studies testing effects of *CycD2* ectopic expression in *Arabidopsis* showed that cDNA transgene could not yield in a full-size cyclin protein because of the internal truncation of the transcript from cDNA (Qi and John, 2007). Only the ectopic expression of the genomic form of *CycD2* resulted in full-length mRNA, increase of CDK/cyclin enzyme activity, reduced cell size at S phase and abundance of G1-phase cells (Healy *et al*., 2001). However, this problem may concern only the *CycD2*;1 protein but not the others that were overexpressed in this experiment (Arp Schnittger, personal communication).

It is also possible that an overproduction of cell cycle proteins resulting from the overexpression will trigger protein degradation processes. Proteolysis was found to be a regulatory pathway which triggers a rapid degradation of target proteins providing an irreversible mechanism that drives the cell cycle progression (Genschik *et al*., 1998). Among components of the plant cell cycle, cyclins, such as A-, B- (Genschik *et al*., 1998), D- (Planchais *et al*., 2004), are subject to extensive regulation by proteolysis. Especially, CycD3;1 was found to be a highly unstable protein whose proteolysis is mediated by a proteasome-dependent pathway. Other cell cycle regulators degraded by this pathway are E2Fc (del Pozo *et al*., 2002) or KRP2 (Verkest *et al*., 2005). Recently, Arabidopsis RBR1 and E2Fs proteins were reported to be regulated by proteasome-dependent degradation during sucrose starvation (Hirano *et al*., 2011). Thus, the overexpression of cell cycle components may lead to the activation of the protein degradation mechanism.

Beside cyclins and CAKs, the activity of the plant CDKs is regulated by additional factors. Among these, seven *Arabidopsis* Kip-Related proteins (KRPs) (Vandepoele *et al*., 2002) are negatively regulating different CDK/cyclins complexes in a tissue specific manner (De Veylder *et al*., 2001) and they might prevent phosphorylation of the RBR protein. In Arabidopsis all seven KRP proteins were shown to interact with D cyclins (Wang *et al*., 1998; Lui *et al*., 2000; De Veylder *et al*., 2001). The KRP2 was found to interact with CDKA;1 (Verkerst *et al*., 2005). The inhibition of the cell cycle caused by the *KRP* overexpression was complemented by co-overexpression of D-type cyclins (Zhou *et al*., 2003). Thus, we performed crosses of mutant lines overexpression the G1/S phase genes with Arabidopsis *KRP* knock-out mutants in order to test if a deficiency of the cell cycle negative regulators may induce parthenogenesis. Microscopic observation revealed that majority of observed ovules contained the normal wild type embryo sacs

without developing zygotes. However, we observed in at very low frequency ovules containing structures resembling multiple egg cell nuclei. These nuclei were smaller than the normal egg cell. We hypothesized that they may result from mitotic divisions of the egg cell nucleus without a replication of the genetic material. Such abnormal mitotic divisions without chromosomal replication after meiosis II were observed in the maize *polymitotic* (*po*) mutant (Wolfe and Liu, 1999).

Plants as well animals display a high complexity of the cell cycle machinery. Components of the cell cycle, their functions and transcriptional regulation have been extensively studied. However, this picture is mainly based on experiments performed on isolated single cells and the role of the cell cycle in the developmental processes still remains elusive. The knowledge of processes like posttranslational modifications, proteolytic degradation of positive and negative regulators is also still limited. Furthermore, the cell cycle mechanism is likely coordinated with other processes of the organism, such as hormonal control, nutrient metabolism, light response, signaling and membrane dynamics, transcriptional regulation or chromatin regulation. In contrast to animals the plant G1 phase appears to be much more influenced by environmental factors (den Boer and Murray, 2000; Gutierrez, 2009). In general, one can conclude that the complexity of the cell cycle mechanisms in plants may cause difficulties in manipulating of genes that are involved in these processes. Reports from overexpression experiments of cell cycle genes under the transcriptional control of the another egg cell specific promoter (egg cell 2) similar to the experiments performed during this PhD work did not lead to the egg cell activation (Arp Schnittger, personal communication). Therefore, one conclusion is that the RBR/E2F/DP pathway is not directly involved in the egg cell activation and initiation of the embryo development in *Arabidopsis thaliana*.

4.2.3. Artificial MicroRNA Targeting *MSI1* and *RBR1* Genes

In this experiment we tested if the silencing of *MSI1* and *RBR1* by artificial microRNAs expressed in the Arabidopsis egg apparatus can trigger parthenogenetic embryo development. The *MSI1* and *RBR1* genes are suggested to be involved in maternal gene networks and epigenetic modification processes in the Arabidopsis egg and the central cells (reviewed in Sundaresan and Alandete-Saez, 2010). The MSI1 protein is required for the FERTILIZATION INDEPERNDENT SEED (FIS) complex

activity (Köhler *et al*., 2003). The complex is involved in both, the early seed development and the repression of seed development in the absence of fertilization. Beside of its role in the FIS complex, MSI1 was found to physically interact with the RBR1 protein (Ach *et al*., 1997; Johnston *et al*.,2008) in a pathway that is known to negatively regulate the expression of the main maintenance DNA methyltransferase *MET1* in the embryo sac (Jullien *et al*., 2008). RBR acts on both, the central and the egg cell, and together with MSI1 binds to the promoter of *MET1*. This results in a decrease of methylation in both egg and central cell nuclear DNA. Mutations in *RBR* lead to an over-proliferation of the embryo sac and initiation of the autonomous endosperm development (Ebel *et al*., 2004). The *msi1* mutants exhibit autonomous endosperm growth and form, at low frequency, parthenogenetic embryos that abort at very early stages of development (Guitton *et al*., 2004; Guitton and Berger, 2005). This is an indication that *MSI1* may be important for repressing autonomous divisions of the egg cell. We assumed that silencing of the *MSI1* transcript restricted only to the egg apparatus may also result in the parthenogenetic embryo development. However, in contrary to *msi1* loss-of-function mutants, the parthenogenetic embryo should proceed to more advanced phases since *MSI1* functions are not disrupted during the embryonic development.

The microRNAs (miRNA) are approximately 21-nucleotide-long single-stranded RNA molecules found in metazoans, animals and plants. They regulate target gene expression by mRNA destabilization or by inhibition of protein translation (Pillai *et al*., 2007). The endogenous miRNA precursor sequences can be modified to create the amiRNA directed against any gene of interest (Schwab *et al*., 2006). Natural miRNAs were shown to be highly specific (Schwab *et al*., 2005, 2006), and their endogenous sequences can easily be modified to deregulate the expression of single or multiple highly conserved genes *in planta* without affecting the expression of other genes. An artificial microRNA can be generated by using the amiRNA designer interface WMD (Schwab *et al*., 2006; Ossowski *et al*., 2008). This web application facilitates the design of suitable amiRNA sequences for different plant species and also supports the design of primers needed for the endogenous miRNA modification. The amiRNAs have been used for efficient gene silencing in various species (Alvarez *et al*., 2006; Niu *et al*., 2006; Schwab *et al*., 2006; Warthmann *et al*., 2008; Khraiwesh *et al*., 2008).

We did not observe the parthenogenetic embryo development in lines expressing the amiRNA targeting *MSI1* gene in the egg apparatus. However, there were no experiments performed to determine whether the *MSI1* transcript is affected by the amiRNA. We cannot, therefore, conclude that the amiRNA is functional and if it is capable to down-regulate its target. Furthermore, it is also recommended for experimental work to select at least two amiRNAs that bind the mRNA of their target gene at different locations, since the secondary structure is thought to influence the miRNA efficacy. Based on these preliminary experiments we cannot draw any conclusions regarding the role of *MSI1* in the parthenogenetic embryo development. We need to complete the experimental work, confirming a reduced abundance of the *MSI1* product by RT-PCR, or testing effectiveness in *MSI1* transcript degradation of another amiRNA. In contrast to *MSI1*, mutant plants expressing amiRNA targeting *RBR1* could not be recovered.

4.3 Outlook

Attempts to dissect the molecular pathways involved in apomixis development in Angiosperms include: (i) studies of the genetic control in natural apomicts and a map-based cloning of genes associated with this trait (ii) identification of genes required for sexual reproduction and a characterization of mutants of the sexual plant displaying elements of apomixis (iii) introgression or engineering of apomixis via classical breeding into sexual relatives. The molecular mechanisms controlling apomictic reproduction remain unclear and introduction of apomixis into crops of agronomical importance has so far been unsuccessful. As in other efforts undertaken to induce elements of apomixis, during this PhD studies the approaches taken to trigger aspects of parthenogenesis did not lead to the identification of novel genes involved in this developmental stages. The random gene activation tagging or mis-expressing of candidate gene in the Arabidopsis egg apparatus did not lead to the parthenogenetic embryo development in absence of fertilization. However, recent reports showed that genetic engineering technologies may allow constructing apomixis *de novo* and that clonal reproduction can be engineered in a sexual plant by manipulating two or four conserved genes that control meiosis and chromosome segregation (Marimuthu *et al*., 2011). It remains crucial to bring more insight into apomictic processes and characterize the genetic pathway regulating this interesting trait.

It may be possible that the chances to trigger the seed development may be higher in the apomeiotic background. Recently identified mutants exhibiting apomeiosis could facilitate future attempts to engineer parthenogenesis. The triple apomeiotic *MiMe* mutant appears to be the most suitable for the purpose of an inducible activating tagging screen. The *MiMe* was shown to be fully penetrant. This mutant line produces viable pollen. Thus, *MiMe* should be introduced into the conditional male sterile background (*dde2-2* line) that the screen for silique elongation will be possible. Combined with optimized induction methods, this approach taken remains a powerful tool in order to tackle the decades old milestone in plant reproduction and breeding. The extensive knowledge and molecular tool available in Arabidopsis allow an adjustment of the setup according to the gained insights.

In future experiments, the mis-expression of candidate genes in the egg cell in the attempt to trigger parthenogenesis could be carried out by using a different promoter. This new promoter should show higher egg cell specificity. Recently published data indicated members of the RKD (RWP-RK domain-containing) transcription factor family (Kòszegi *et al*., 2011) as the egg cell specific promoters. The RKD transcription factors were shown to be capable to induce a subset of the egg transcription profile in sporophytic cells, causing a reprogramming process.

Achieving an inactivation of the RBR1 protein by overexpression of genes activating the G1/S transition was complicated due to the complexity of the cell cycle. Attempts to activate the egg cell divisions by the overexpression of genes promoting the G1-to-S phase transition should focus on direct silencing of the *RBR1* gene. The vector containing an artificial micro RNA targeting *RBR1* gene was generated during this PhD studies. Thus, generation of tranformants and microscopic observation of the female gametophyte of these lines should be the next step.

5. ACKNOWLEDGEMENTS

I want to thank Prof. Dr. Ueli Grossniklaus for giving me the opportunity to work in his lab, his support and supervision during my PhD studies.

I am grateful to Dr. Mark D. Curtis for initiation the project and introducing my to the lab work.

I would like to thank Prof. Dr. Charles Spillane from Centre for Chromosome Biology at NUI Galway for agreeing to be my external supervisor, a part of the PhD committee and reviewing my thesis.

Many thanks also to Lukas for his help at the beginning of my studies and for helpful comments on the manuscript.

I want to thank all the current and former members of the Grossniklaus-group including the Medea Club for helpful discussions, comments and a very friendly atmosphere.

Special thanks go to the P2-27 members. It was a pleasure to work in this lab.

For technical assistance, lab organization I want to thank Valeria Gagliardini, Daniela Guthörl, Christof Eichenberger and Arturo Bolanos. I thank Peter Kopf for supply of media and solutions. Further I thank also Alessio Grossi for his help during the screen.

Zürich, July 2012

6. APPENDIX

6.1. Publications

Female Gametophytic Mutants of Arabidopsis thaliana Identified in a Gene Trap Insertional Mutagenesis

Int. J. Dev. Biol. 55: 73-84 (2011)
doi: 10.1387/ijdb.092989vb

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Female gametophytic mutants of *Arabidopsis thaliana* identified in a gene trap insertional mutagenesis screen

VLADIMIR B. BRUKHIN*,#,1, MILOSLAWA JACIUBEK[1], ARTURO BOLAÑOS CARPIO[1], VERA KUZMINA[1] and UELI GROSSNIKLAUS[1]

[1]*Institute of Plant Biology and Zürich-Basel Plant Science Center, University of Zürich, Switzerland*

ABSTRACT In plants, the male and female gametophytes represent the haploid generation that alternates with the diploid sporophytic generation. Male and female gametophytes develop from haploid micro- and megaspores, respectively. In flowering plants (angiosperms), the spores themselves arise from the sporophyte through meiotic divisions of sporogenous cells in the reproductive organs of the flower. Male and female gametophytes contain two pairs of gametes that participate in double fertilization, a distinctive feature of angiosperms. In this paper, we describe the employment of a transposon-based gene trap system to identify mutations affecting the gametophytic phase of the plant life cycle. Mutants affecting female gametogenesis were identified in a two-step screen for (i) reduced fertility (seed abortion or undeveloped ovules) and (ii) segregation ratio distortion. Non-functional female gametophytes do not initiate seed development, leading to semi-sterility such that causal or linked alleles are transmitted at reduced frequency to the progeny (non-Mendelian segregation). From a population of 2,511 transposants, we identified 54 lines with reduced seed set (2%). Examination of their distorted segregation ratios and seed phenotypes led to the isolation of 12 gametophytic mutants, six of which are described herein. Chromosomal sequences flanking the transposon insertions were identified and physically mapped onto the genome sequence of *Arabidopsis thaliana*. Surprisingly, the insertion sites were often associated with chromosomal rearrangements, making it difficult to assign the mutant phenotypes to a specific gene. The mutants were classified according to the process affected at the time of arrest, i.e. showing mitotic, karyogamic, maternal or degenerative phenotypes.

KEY WORDS: *Ds transposon, female gametophyte, segregation ratio distortion, sexual plant reproduction*

Introduction

It is very difficult, often impossible, to identify and characterize genes involved in basic cellular processes in diploid higher eukaryotes as organisms with a homozygous mutation in an essential gene are lethal. The study of haploid plant gametophytes provides an excellent opportunity to examine such genes essential to cell division or other fundamental cellular processes. Heterozygous recessive mutations are not lethal to the diploid tissues of the sporophyte, such that their lethal phenotype can be characterized in 1/2 of the haploid gametophytes that are produced by a plant heterozygous for a gametophyte lethal mutation.

In seed plants the life cycle alternates between a dominant diploid phase (sporophyte) and a strongly reduced haploid phase (gametophyte). In angiosperms, the haploid female gametophyte (embryo sac) is generated by the functional megaspore, the only surviving meiotic product, through three mitotic divisions. The female gametophyte typically consists of only seven cells, two female gametes (egg and central cell) and five accessory cells. It is highly inaccessible because it develops inside the ovule, deeply embedded within the gyneocium of the flower (Grossniklaus and Schneitz, 1998; Drews *et al.*, 1998, Yadegari and Drews, 2004). The male gametophtye (pollen) is even more reduced and usually consists of only three cells, two of which are sperm cells. Seed development is initiated by double fertilization, where the pollen

Abbreviations used in this paper: Ac, Activator; Ds, Dissociation; DIC, differential interference contrast; GUS, β-glucoronidase; GT, gene trap; TAIL-PCR, thermal asymmetric interlaced polymerase chain reaction; iPCR, inverse polymerase chain reaction.

***Address correspondence to:** Vladimir B. Brukhin. Aberystwyth University, IBERS, Edward Llwyd Building, Penglais, Aberystwyth, Ceredigion SY23 3DA, UK. Fax: +44-1970-62-2350. e-mail: vlb@aber.ac.uk #Current address: Institute of Biological, Environmental and Rural Sciences, University of Aberystwyth, UK

Accepted: 27 July 2010. *Final author corrected PDF published online:* 21 January 2011. *Edited by:* Mieke Van Lijsebettens

ISSN: Online 1696-3547, Print 0214-6282

tube enters the embryo sac and releases the two sperm cells. One sperm cell fuses with the haploid egg cell to produce a diploid zygote, which initiates embryo development and constitutes the next sporophytic generation. The second sperm cell fuses with a homo-diploid central cell, initiating the development of the triploid endosperm that provides nourishment to the developing embryo (Olsen, 2004). This complex developmental process is unlike any found in animals, where the meiotic products directly differentiate into the gametes. In contrast, the meiotic products of plants undergo several mitotic divisions to form a multicellular haploid organism with distinct cell types, including the two pairs of gametes. This collection of haploid cells provides a short window of opportunity to identify genes involved in cellular processes fundamental to their survival or playing a role in their specific developmental regulation. A viable diploid sporophyte heterozygous for a mutated essential gene will produce 50% viable (wild-type) haploid gametophytes and 50% abnormal (mutant) haploid gametophytes. This unique feature of plants allows genes essential to an organism's survival to be dissected, since mutants are characterized by the developmental stage at which they are arrested. *Arabidopsis thaliana* is the organism of choice to study such lethal mutations since it has a short life cycle, excellent tools for forward and reverse genetics, and produces a large number of gametophytes (reviewed in Page and Grossniklaus, 2002).

Up to the mid 1990s few molecular or genetic studies had been made on female gametophyte development and only a single female gametophytic mutant had been described in *Arabidopsis* (Redei, 1965). This lack of knowledge led Jack Heslop-Harrison to suggest that the gametophyte was "the forgotten generation" (Heslop-Harrison, 1979). Over the last two decades emerging new genetic and bioinformatics tools have allowed exploiting a functional genomics approach to dissect female gametophyte development and to identify the specific genes involved. Significant progress has been made using insertion mutagenesis to provide tools for forward and reverse genetic studies. For instance, valuable mutant populations of *Arabidopsis* have been generated, using systems designed to create low copy number transposon insertions at random positions in the genome. Such systems provide a physical tag that pinpoints the location of a mutation, which can be used for mapping and as a starting point from which to sequence an insertion site (e.g. Sundaresan *et al.*, 1995, Springer *et al.*, 1995; Moore *et al.*, 1997, Parinov *et al.*, 1999, Zhang *et al.*, 2003, van Enckevort *et al.*, 2005). During the last few years new gametophytic mutants from large-scale insertional mutagenesis projects – using either transposons or T-DNA insertions – have been isolated and loci essential for male and female gametophyte development identified (Moore *et al.*, 1997; Feldmann *et al.*, 1997; Howden *et al.*, 1998; Bonhomme *et al.*, 1998; Christensen *et al.*, 1998, 2002; Grini *et al.*, 1999; Moore, 2002; Lalanne *et al.*, 2004; Pagnussat *et al.*, 2005; reviewed in Brukhin *et al.*, 2005a). Nevertheless, the identification of the developmental pathways and genes that orchestrate female gametophyte function and developmental progression are far from being accomplished.

We have exploited the *Activator/Dissociation* (*Ac/Ds*) gene trap transposon system developed by Sundaresan and colleagues (1995) to generate new insertions distributed throughout the genome of *Arabidopsis*. These insertion lines were screened to identify essential genes affecting gametophyte development or function, and their phenotypes were analyzed. Mutations affecting the gametophytic phase of the life cycle were identified in a two-step screen for (i) reduced fertility (seed abortion or undeveloped ovules) and (ii) a distorted segregation ratio of the dominant marker present on the *Ds* transposon (for details on this strategy, see Moore *et al.*, 1997; Page and Grossniklaus, 2002).

Here we provide a detailed description of six gametophytic mutants showing a stably distorted segregation ratio of the dominant marker present on the *Ds* element and reduced fertility or a semi-sterile phenotype. We provide the genetic and cytological characteristics of these mutants. Based on the cytology of terminally arrested embryo sacs, we classified them as mitotic, karyogamic, maternal, or degenerative phenotypes. For all mutants we describe the phenotype at the morphological and cytological level, genetic segregation, and transmission efficiencies through both male and female gametophytes. Despite a tight co-segregation of the *Ds* elements with the mutant phenotypes, only one of the mutants could be unambiguously assigned to a gene encoding a TATC-like protein, while two are likely associated with rearrangements, and the final three are apparently not tagged as assessed by the analysis of additional insertion alleles. This raises important questions as to the use of insertional mutagenesis in combination with segregation ratio distortion to identify gametophytic mutants.

Results and Discussion

Insertional mutagenesis creates mutations affecting plant reproduction

We generated 2511 gene trap (GT) lines, each with putatively a single, independent *Ds* insertion, and screened the progeny derived from self-fertilization of these lines for gametophytic mutants. From all GT lines generated we found 54 (2%) lines with a reduced seed set.

Deviation from a 3:1 Mendelian segregation ratio of kanamycin resistant (Kan^r) to sensitive (Kan^s) seedlings towards Kan^r/Kan^s ratios of less than 2:1 is a characteristic of mutations affecting one or both gametophytes (Feldmann *et al.*, 1997; Moore *et al.*, 1997; Howden *et al.*, 1998). We screened the entire GT population for a distorted segregation ratio of the Kan^r gene present on the *Ds* element. 31% of the GT lines produced seedlings of which all or nearly all were resistant to kanamycin. This could be due either to the homozygosity of the *Ds* insertion or due to the insertion of more than one *Ds* element per line. 40% of the lines showed Kan^r:Kan^s segregation ratios of about 3:1, corresponding to a normal Mendelian segregation ratio. 19.5% of the lines gave reduced segregation ratios in the range 2:1 to 2.5:1, which are likely to result from a recessive, zygotic embryo lethal phenotype. 9.5% of the GT lines showed distorted segregation ratios between 2:1 and 0.14:1. The latter might be caused by a partial or full reduction of transmission of the *Ds* insertion through one or both sexes. In order to learn which of the semi-sterile GT lines showed ovule and/or seed abortion due to reduced transmission through male and/or female gametophytes, we re-examined 54 pre-selected lines with a reduced seed set for segregation ratio distortion. Finally, we identified 12 GT lines for further investigation, which combined a semi-sterile phenotype with a segregation distortion ratio that matched the prediction for a gametophytic mutation. These lines correspond to 0.48% of all lines screened.

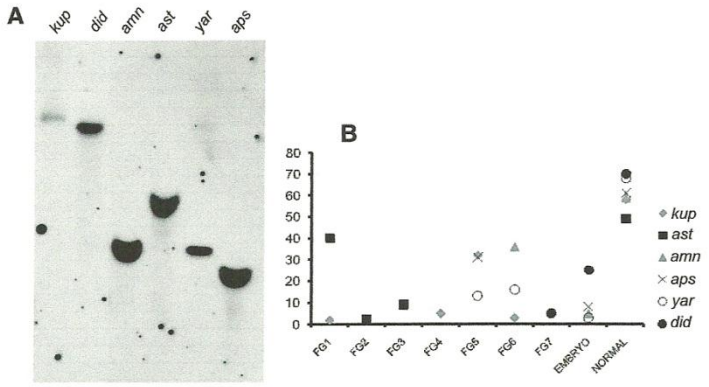


Fig. 1. Confirmation of a single *Ds* insertion per line and summary of the phenotypic characterization of six mutants affecting gametophyte development. (A) *Genomic Southern blot indicates the presence of a single* Ds *element in each mutant tested. For Southern blotting genomic DNA of the respective mutants was restricted with* Eco*RI endonuclease and a digoxygenin-labelled 5'* Ds *probe was used to detect the left border fragment.* **(B)** *The graph summarizes the percentage of ovules with a terminal phenotype at a particular stage of development for all six mutants. X-axis – stages of female gametophyte at which arrested gametophytes were found. Y-axis – percentage of arrested ovules/seeds at each stage and normal seeds. FG1, one-nucleate female gametophyte; FG2-F3, two-nucleate female gametophyte; FG4, four-nucleate female gametophyte with unfused polar nuclei; FG6, seven-celled female gametophyte with polar nuclei fused; FG7, four-celled female gametophyte: the antipodal cells have degenerated; FG8, three-celled female gametophyte: one of the synergid cells degenerates and the mature female gametophyte consists of the egg cell, the central cell, and one persistent synergid cell; stages after Christensen* et al. *2007.*

This frequency is within the range obtained in other screens aimed at identifying gametophytic mutations (Feldmann *et al.*, 1997; Moore *et al.*, 1997; Howden *et al.*, 1998; Bonhome *et al.*, 1998; Lalanne *et al.*, 2004; Pagnussat *et al.*, 2005).

In this study, we report on the six of these mutants with phenotypes predominantly affecting female gametophyte development or function. We named these mutants after gods or goddesses from various Pantheons whose influence is exerted, according to legend, upon fertility and reproduction.

Gametophytic mutants generally affect transmission through both sexes

Self-crossed progeny of all six mutants showed stable segregation ratio distortion over at least three to four generations, suggesting that the mutations in the lines under study were associated with the *Ds* insertion. However, a distortion in the segregation ratio among the progeny from a self-cross does not provide insights into which sex is affected by the mutation. Although we specifically focused on mutants that showed reduced fertility – and thus expected them to have a defect in female gametophyte development or function – these mutants may also affect the male gametophyte to some extent.

To quantify the transmission efficiency through either sex we measured the *Ds* inheritance in reciprocal crosses to the wild type. Southern blot analysis was carried out to investigate whether the genome of each GT line did contain only one *Ds* element, as

TABLE 1

PHENOTYPE, GENETIC SEGREGATION AND TRANSMISSION ANALYSIS OF THE GAMETOPHYTIC MUTANTS

Mutant	Phenotype	Seeds Scored	Segregation Kan^r: Kan^s	Seeds Scored	P-Value	TEF	Seeds Scored	TEM	Seeds Scored	*Ds* insertion site	Disrupted gene function
Kupalo	58% N+42% UO	1302	1.04:1	161	0.81	40%	424	68%	275	At2g01070	PTM1-like protein
Astlik	49%N+51%UO	570	1.66:1	187	0.23	61%	46	98%	107	Deletion of At3g03030 At3g03040 At3g03050 At3g03060 *Ds* 3'-end At1g75990	 F-box family protein F-box family protein Cellulose synthase-like protein AAA-type ATPase family protein AAA-type ATPase family protein RPN3b subunit of 26S proteasome EMBRYO DEFECTIVE 2719
amon	59%N+36%UO+5%A	1030	1.13:1	194	0.39	56%	426	24%	31	At4g02700	
apis	61%N+31%UO+8%A	1045	0.32:1	212	NA	5%	113	8%	232	At5g44520	
yarilo	68%N+29%UO+3%A	1221	0.30:1	341	NA	15%	109	9%	146	*Ds* 5'-end At2g34680	AIR9 auxin-induced in root cultures
Didilia (apg2)	70%N+5%UO+25%A	1010	1.05:1	254	0.71	34%	270	59%	195	At2g01110	TATC-like protein, UNFERTILIZED EMBRYO SAC3
wt	93%N+6%UO+1%A	844[a]	3:1[b]		-	100%		100%		---	

[a]based on data from Moore *et al.*, 1997

[b]expected, ideal values

N- normal seeds; A- aborted seeds; UO- undeveloped ovules. A P-value of ≥0.05 based on an expected 1:1 Kanr: Kans segregation ratio for a gametophytic lethal mutation. NA, not applicable for the mutants *yar* and *aps* where segregation ration was much less than 1:1 due to defective transmission through both sexes. Transmission efficiencies were calculated according to Howden *et al.* (1998): TE=Kan^r–/ Kan^s x 100%; Kan^r, kanamycin-resistant seedlings; Kan^s, kanamycin-sensitive seedlings; TEF- female transmission efficiency, TEM- male transmission efficiency.

multiple insertions would confound the analysis. As shown in Fig. 1A each of the selected six lines contained only a single *Ds* element in its genome. Linkage analysis of 100 to 250 plants from the progeny of each line tested revealed complete co-segregation of the *Ds* element (i.e. Kan^r) and the mutant phenotype. These findings indicate that the insertions were either responsible for the observed semi-sterile phenotype or were tightly linked to a mutation responsible for the phenotype.

The efficiency of *Ds* transmission was estimated according to Howden and colleagues (1998). As shown in Table 1, all insertions showed a reduced transmission through the female gametophyte (TEF, transmission efficiency female) as expected, ranging from 5% to 61% of the wild-type allele. However, except for one mutant (*astlik*), all others showed also a reduced transmission of *the Ds* through the male gametophyte (TEM, transmission efficiency male) ranging from 8% to 68% of the wild-type allele. The severity of reduced transmission through male and female gametophytes is correlated (correlation coefficient R=0.72), indicating that likely some basic cellular functions are affected in these mutants rather than sex-specific developmental processes. Furthermore, segregation analysis of self-crosses of all six mutants did not produce any homozygous plants. This indicates that, in addition to the gametophytic defects revealed in heterozygotes, homozygous mutants are likely zygotic lethal.

In summary, the gametophytic mutants analyzed here generally affect both male and female gametophytes, confirming earlier findings that sex-specific gametophytic mutants are rare (Moore *et al.*, 1997; Feldmann *et al.*, 1997; Howden *et al.*, 1998; Bonhomme *et al.*, 1998; Christensen *et al.*, 1998, 2002; Grini *et al.*, 1999; Moore, 2002; Lalanne *et al.*, 2004; Boavida *et al.*, 2009). This is expected since many events during the process of male and female gametophyte formation are similar and the expression of many essential genes during the haploid phase will be required in both gametophytes.

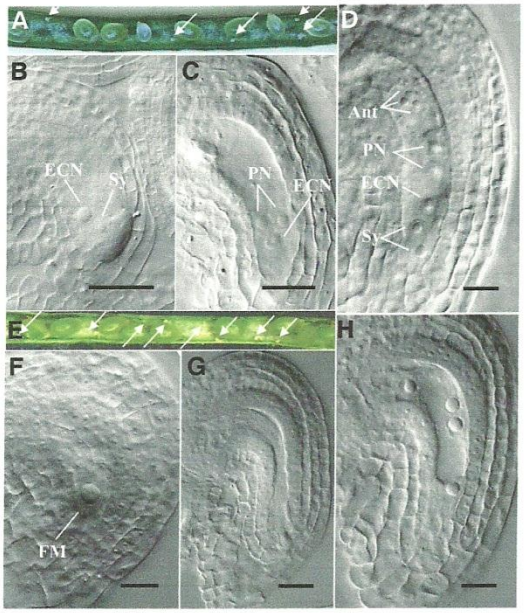


Fig. 2. Embryo sac and seed set phenotypes of the mitotic class mutants *kupalo* and *astlik*. **(A-C)** kupalo, **(D)** *wild-type*, **(E-H)** astlik. **(A)** *Silique containing normal seed and infertile ovules (arrows).* **(B)** *Defective synergid cell.* **(C)** *Unsuccessful cellularization of the embryo sac with missing synergid nuclei.* **(D)** *Seven-celled, 8-nucleate wild-type embryo sac containing an egg cell, two synergids, two un-fused polar nuclei, and three antipodal cells.* **(E)** *Silique containing normal seeds and infertile ovules (arrows).* **(F)** *Arrested functional megaspore.* **(G)** *Arrested ovule lacking an embryo sac but with continued integument growth.* **(H)** *Embryo sac arrested at the four-nucleate stage. ECN, egg cell nucleus; FM, functional megaspore; PN, polar nuclei; Ant, antipodals; Sy, synergids. Bars, 10 μm.*

The kupalo *and* astlik *mutants are defective in mitotic progression*

Two mutants were categorized into the class showing mitotic phenotypes, which are defective in one or several of the three mitotic divisions (FG1 to FG5, according to Christensen *et al.*, 1997) that form the mature embryo sac (Brukhin *et al.*, 2005a). The first mutant was named *kupalo (kup)*, after the Slavic god of herbs, sorcery, sex, and midsummer. The segregation ratio of Kan^r:Kan^s seedlings derived from *kup/KUP* plants was 1.04:1 (Table 1). The transmission of *Ds* through the female gametophyte was only 40%, while male transmission was reduced to 68% (Table 1). Analyses of the *kup* mutant phenotype showed that in mature siliques 42% of ovules were unfertilized (Table 1), remaining white and small without developing into a seed (Fig. 2A, arrows). Cytological observations under differential interference contrast (DIC) optics revealed that the majority of ovules (32%) arrested at stage FG5 (Fig. 2B). At this stage, in wild-type ovules the syncytial, eight-nucleate embryo sac cellularizes to form the seven-celled female gametophyte with three antipodals, two synergids, an egg cell, and a bi-nucleate central cell (Fig. 2D). 5% of the ovules were arrested at FG3-FG4, i.e. the mutant showed pleiotropic defects during the nuclear division phase between the two- and eight-nucleate stage. 2% of the embryo sacs were arrested at FG1 during the first mitotic division of the functional megaspore, and 3% of ovules reached FG6, which corresponds to the mature embryo sac (Fig. 1B), but they did not progress beyond. Interestingly, some ovules in *kup* showed embryo sacs with defective (Fig. 2B) or missing (Fig. 2C) synergids and abnormalities in the polar nuclei (Fig. 2C). Analysis of the mature pollen stained with DAPI indicated that about 12% of the pollen grains were abnormal in comparison to 5% in the wild type (Table 2). Defective mature pollen grains had only two nuclei, consistent with a defect in mitotic progression. Defective pollen showed either one weakly stained, vegetative nucleus and one strongly stained, single sperm nucleus, or two weakly stained, elongated nuclei (not shown).

In the *kup* mutant, the DNA fragment flanking the *Ds* element corresponded to the second exon of the *At2g01070* gene (Fig. 3), which encodes a PTM1-like trans-membrane protein of unknown molecular function (Table 1). This protein is reported to co-purify with late Golgi vesicles, which contribute to cell wall and membrane formation and also deliver regulatory substances; consequently the disruption of such a gene may affect membrane

properties and cellularization, a process blocked in *kup* mutant embryo sacs. Only one other insertion within the *At2g01070* gene was available, but it showed no obvious fertility defects (not shown). Since we could not confirm the T-DNA insertion site in this second line, it is currently unclear whether the *Ds* disrupting *At2g01070* is indeed responsible for the *kup* phenotype.

The mutant *astlik (ast)* was named after the Armenian goddess of love and fertility. The Kan^r:Kan^s segregation ratio in the progeny of *ast*/*AST* plants was 1.66:1 (Table 1), i.e. characteristically a combination of zygotic and gametophytic lethality. Female transmission efficiency was reduced to 61% (Table 1), while male transmission was similar to that of wild-type plants. In mature siliques of the *ast* mutant 51% of ovules were unfertilized (Fig. 2E, arrows). Most of these (40% of the total) were arrested prior to the first mitotic division of the functional megaspore at FG1 (Fig. 2F), while a small number arrested at FG2 (2%) or FG3 (9%) - a stage corresponding to the four-nuclear embryo sac (Fig. 2H, Fig. 1B). Despite these abnormalities, the integuments continued to grow and the defective ovules in mature siliques often appeared as shown in Fig. 2G, where no embryo sac can be discerned within the ovule. Assessment of pollen grains in the *ast*/*AST* mutant showed that 10% of the mature pollen grains were abnormal with phenotypes similar to those found in *kup* (Table 2).

In *ast* the 5'-end of the *Ds* was inserted in the sixth exon of the F-box protein gene *At3g03030* and 3'-end was found in the fifth exon of *At3g03060* encoding a protein similar to the AAA-type ATPase family of proteins (Bayer *et al.*, 2006), thereby probably deleting two other genes, *At3g03040* and *At3g03050* (Fig. 3). It is therefore not clear which of the deleted gene(s) might be responsible for the phenotype. To possibly link the phenotype to one of the affected genes, we assessed several additional insertion mutants. Exonic T-DNA insertions in *At3g03030* (SALK_094197) and *At3g03040* (SALK_056065 and SALK_056153) showed no obvious phenotypes. Conversely, an exonic *Ds* insertion in *At3g03050* showed a slight reduction in seed set with about 10% unfertilized ovules (Table 1S). *At3g03050* encodes a cellulose synthase-like protein, mutations in which cause root hairs to rupture at their tip soon after initiation (Bernal *et al.*, 2008). This gene is expressed in the embryo sac as shown by genetic subtraction profiling (Johnston *et al.*, 2007). However, two other exonic insertions in *At3g03050* showed no fertility phenotype (not shown), making it unlikely that this gene is responsible for the *ast* phenotype. Disruption of the *At3g03060* promoter region (SALK_1304_B10) was associated with 8% unfertilised ovules and 15% aborted seeds in mature siliques (Table 1S). This gene encodes a protein of the AAA-type ATPase family (Bayer *et al.*, 2006). However, since a second exonic T-DNA insertion shows no obvious phenotype (not shown), *At3g03060* is probably not responsible for gametophytic lethality in the *ast* mutant.

In addition to the small deletion on chromosome 3, we found a 3'-end of *Ds* on chromosome 1 in the sixth exon of *At1g75990*, encoding the 26S proteasome regulatory subunit RPN3b, which participates in ubiquitin-dependent protein catabolic processes (Vierstra, 2003; Smalle and Vierstra, 2004; Yang *et al.*, 2004) essential to gametophyte development (Gallois *et al.*, 2009) and the proper execution of mitosis in all eukaryotes (Den Elzen and Pines, 2001; Vierstra 2003; Smalle and Vierstra, 2004; Yang *et al.*, 2004, Brukhin *et al.*, 2005b). Given that we could not amplify the 5' end of the *Ds*, that only a single *Ds* element was detected in this line (Fig. 1A), and that the segregation ratio of Kan^r is consistent with a single insertion (Table 1), we conclude that only a short piece of the 3'-end of the *Ds* is present on chromosome 1. In contrast the *Ds* insertion on chromosome 3 appears to be associated with a deletion, as was reported for several other lines that were generated using this transposon system (Oh *et al.*, 2003; Page *et al.*, 2004, Boavida *et al.*, 2009).

Given the complex rearrangements observed in *ast*, it is not yet possible to assign the defect to a particular gene. However, because the mutant shows a mitotic arrest, it may well be that the mutation in *RPN3b* plays a major part in the *ast* phenotype. Although individual insertions into *At3g03030* and *At3g03040*, which both encode F-box proteins, have no obvious phenotypes, their simultaneous disruption may contribute to the mitotic class phenotype seen in *ast* mutants. F-box proteins are components of SCF ubiquitin-ligase complexes that function in binding substrates for ubiquitin-mediated proteolysis (Kipreos and Paqano, 2000). Ubiquitin-mediated removal of regulatory proteins controlling the cell cycle is important for normal mitotic progression and their disruption may thus lead to mitotic arrest, possibly in conjunction with the disruption of *RPN3b* in the *ast* mutant.

The amon *and* apis *mutants affect karyogamy*

Mutants of the karyogamy class affect the fusion of polar nuclei. During normal development, these nuclei migrate from opposite poles of the embryo sac towards the centre, where they fuse prior to fertilization to form the homo-diploid nucleus of the central cell. A characteristic of many karyogamic mutants is a gametophytic maternal-effect followed by seed abortion. In the

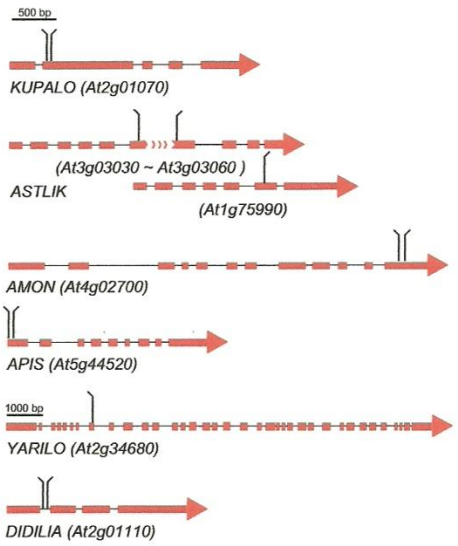


Fig. 3. Schematic representation of *Ds* element insertion sites. *The intron-exon structure of the disrupted genes is shown. 3'- and 5'-ends of the inserted* Ds *elements are indicated by vertical lines ticked to the left and right, respectively.*

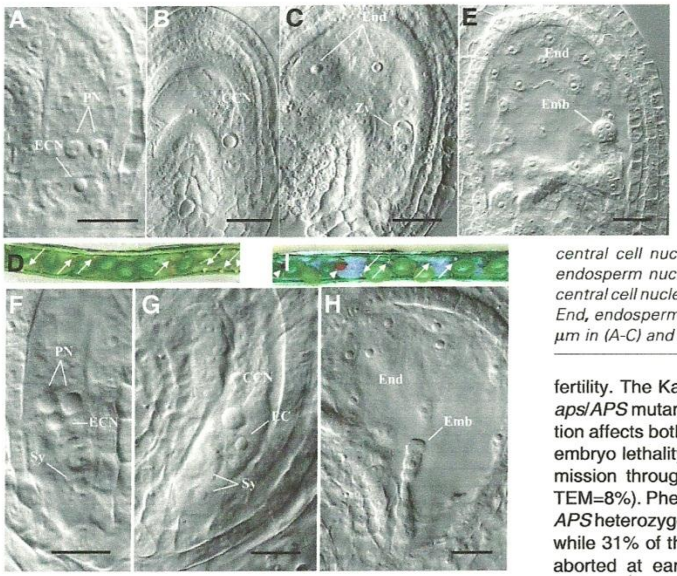


Fig. 4. Embryo sac and seed set phenotypes of the karyogamy class mutants *amon* and *apis*. **(A-E)** amon, **(F-I)** apis. **(A,F)** *Un-fused polar nuclei in the central cell.* **(B)** *Degenerating central cell nucleus.* **(C)** *Degenerating zygote, which is elongated but did not divide, surrounded by abnormal endosperm nuclei.* **(E)** *Normal wild-type seed with octant embryo and alveolar endosperm - seed from the same silique as the defective ovules shown in (B,C).* **(D, I)** *Siliques containing normal seed, infertile ovules (arrows), and aborted seeds (arrowheads) that arrested shortly after fertilization.* **(G)** *Unusually large central cell nucleus.* **(H)** *Shrunken, degenerating two-cell embryo, endosperm nuclei were uniformly small and undifferentiated. CCN, central cell nucleus; EC, egg cell; ECN, egg cell nucleus; Emb, embryo; End, endosperm; PN, polar nuclei; Sy, synergid; Zy, zygote. Bars, 10 μm in (A-C) and (F-H) and 25 μm in (E).*

present study we report two gametophytic mutants, *amon (amn)* and *apis (aps)*, which fall into this category.

The mutant *amon* was named after the Egyptian god of fertility. Among the progeny of *amn*/*AMN* mutants Kan^r:Kan^s segregates in a ratio of 1.13:1 and transmission efficiency was severely reduced through both the male and female gametophytes (TEF=56%, TEM=24%; Table 1). Mature siliques of heterozygous *amn*/*AMN* plants contained 59% normal seed, 36% white, unfertilized ovules, and 5% brown, early aborted seeds (Fig. 4D). The majority of ovules (over 30%) contained abnormally enlarged, unfused polar nuclei (Fig. 4A) or, if the polar nuclei had fused, the central cell nucleus had initiated degeneration (Fig. 4B). These mutant ovules were found in siliques alongside normal seeds that either contained a mature embryo sac or occasionally an early stage embryo, which had undergone a few divisions together with developing endosperm (Fig. 4E). In summary, the white, unfertilized ovules of *amn* (36%) arrested between stages FG5 and FG6 (Fig. 1B), while the brown, early aborted seed (5%) arrested at the zygotic stage (Fig. 4C) or during embryo development after a few cell divisions (not shown). About 10% of the mature pollen grains displayed a defect: 3% contained only a single nucleus (Fig. 5 B-C), while the remaining 7% had two nuclei, similar to what was observed in *kup* and *ast* (Table 2).

In *amn* the *Ds* element was inserted in the 12-th exon of *At4g02700* encoding a sulfate transporter 3;2 (Fig. 3), which is thought to be responsible for the uptake and translocation of sulphate through the cell membrane (Takahashi *et al.*, 2000) and whose expression is enriched in the embryo sac (Johnston *et al.*, 2007). The assessment of eight additional T-DNA insertions within the coding region of *At4g02700* demonstrated no fertility phenotypes, however, indicating that this gene is not responsible for the *amn* phenotype.

Apis (aps) was named after an Egyptian bull deity representing fertility. The Kan^r:Kan^s segregation ratio among the progeny of *aps*/*APS* mutants was 0.32:1 (Table 1), indicating that the mutation affects both female and male gametophytes and may cause embryo lethality. Indeed, reciprocal crosses showed that transmission through both sexes was strongly reduced (TEF=5%, TEM=8%). Phenotypic assays of seeds in mature siliques of *aps*/*APS* heterozygotes showed that 61% of seeds appeared normal, while 31% of the ovules were unfertilized and 8% of the seeds aborted at early developmental stages (Fig. 4I). Cytological analyses revealed that 31% of the arrested embryo sacs had unfused polar nuclei at stage FG5 (Fig. 4F), or defects soon after central cell formation at stage FG6 (Fig. 4G). 8% of the ovules were arrested and aborted soon after the initiation of seed development (Fig. 4 H,I arrowheads). This might be due to a

TABLE 2

PHENOTYPES IN MATURE POLLEN GRAINS OF GAMETOPYTIC MUTANTS

DAPI fluorescence pattern	*kup*	*ast*	*amn*	*aps*	*yar*	*did*	wt
	n=553	n=525	n=766	n=513	n=661	n=422	n=566
	88.6%	90.3%	90.1%	91.4%	67.2%	92.2%	95.2%
			0.3%		3.3%	0.5%	0.5%
	3.4%	3.2%	2.2%	7.6%		4%	1.5%
	7.6%	6.5%	4.6%	1%	24.2%	2.6%	2.8%
	0.4%		0.1%		0.8%	0.7%	
			2.7%		4.5%		

wt – wild type; n - scored pollen grains. For examples of DAPI fluorescence patterns see Fig. 5.

gametophytic maternal effect leading to embryo lethality when mutant female gametophytes get occasionally fertilized. Alternatively, aborted seeds could correspond to homozygous mutants showing zygotic embryo lethality. The latter explanation is unlikely, however, as less than 0.5% homozygous embryos are expected based on the experimentally determined transmission efficiencies. Therefore, we conclude that the vast majority of aborted seeds are caused by a gametophytic maternal effect of the *aps* mutation. Of the mature pollen grains, 9% percent were abnormal with the majority showing only one large, weakly stained and one small, strongly stained nucleus (Table 2; Fig. 5D).

In the *aps* mutant the *Ds* element was found in the first exon of *At5g44520*, encoding a ribulose 5-phosphate isomerase-related protein (Fig. 3). Scoring five additional T-DNA insertions in *At5g44520* (one exonic, one intronic, and three in the promoter region) revealed no obvious phenotypes. Therefore, the *aps* mutant phenotype is likely not caused by a disruption of this gene.

The yarilo *mutant causes the degeneration of central cell, egg cell and synergids*

The degenerative class of mutants show spontaneous abnormal nuclear degeneration during embryo sac development that has dramatic effects on nuclear stability. The *yarilo* (*yar*) mutant was named after the handsome, youthful Slavic god of passion, sex, and lust. The *yar* mutant causes degeneration of embryo sac components. The Kan^r:Kan^s segregation ratio of 0.3:1 in this mutant indicates an involvement of both gametophytes in the phenotype, as was seen with *aps* (Table 1). Indeed, the transmission efficiency was very low through both sexes (TEF=15%, TEM=9%) (Table 1). Mature *yar* siliques contained 29% unfertilized, arrested ovules and 3% brownish, aborted seeds. Microscopic investigations of the *yar* mutant showed deformation of the polar nuclei especially in the nucleolus, the most visible component of the nucleus in cleared preparations (Fig. 6B). Cytological observations of *yar* demonstrated that 13% of the ovules had large degenerating nuclei, which arrested just prior to the fusion of the polar nuclei at stage FG4–FG5 (Fig. 6A), or soon after fusion at stages FG5–FG6 in 16% of cases (Fig. 6B, Fig. 1B). Often, we also observed degeneration of the egg cell, synergid, and central cell nuclei. A small proportion of the seeds (3%) aborted at the two-cell embryo stage (Fig. 6C). This aspect is characteristic of seed abortion effected by the maternal gametophyte but may also be due to zygotic embryo lethality, which is expected to arise at a frequency of about 1.3%. Gametophytic maternal effects were previously observed as late phenotypes of the karyogamy and degenerative class mutants and they may also be features of the mitotic mutant class. Investigations of pollen behaviour *in planta* revealed that a large number of *yar* pollen grains failed to stick to the mature stigma (not shown). An observation of pollen grains stained with DAPI demonstrated that almost 33% of the pollen grains were defective (Table 2), with only one strongly stained nucleus in nearly 5% and two weakly stained, elongated (possibly degrading) nuclei in 24% of the pollen grains (Fig. 5E), respectively.

In *yar*, the 5'-end of the *Ds* element was found in the eighth exon of the *AIR9* (*AUXIN-INDUCED IN ROOT CULTURES9*) gene (*At2g34680*), which was previously identified in a differential cDNA library screen comparing auxin-treated and non-treated root cultures (Neuteboom *et al.*, 1999). The AIR9 protein associates with microtubules in land plants, where it recognizes the cortical division site during pre-prophase and, later on, the site of cell plate formation during cytokinesis (Buschmann *et al.*, 2006). A Meta-Profile analysis of gene expression using Genevestigator tools (Hruz *et al.*, 2008) indicates that *At2g34680* is highly expressed in inflorescence meristems, flowers, stamens, carpels, roots of the mature plant, and at the young rosette, bolting and flowering stages of developing plants. The β-glucuronidase (GUS) reporter present on the *Ds* transposon in *yar* shows strong expression in the roots of young seedlings about two weeks after germination (Fig. 6I), while other vegetative or generative parts of mutant plants showed no GUS activity.

Assessment of additional insertion lines revealed a complex situation, with 3 lines (two exonic, and one in the promoter region) having no obvious phenotype, but one line showing reduced fertility similar to *yar*. A T-DNA insertion (SALK_113675) in the intron of *At2g34680* showed siliques with 22.7% unfertilized ovules and 1.3% aborted seeds in heterozygous plants (Table 1S). In a screen for mutants affecting male progamic development the same gene was disrupted in the *ungud9* (*ung9*) mutant, causing reduced male and female transmission efficiencies of 4.2% and 55.3%, respectively (Lalanne *et al.*, 2004). Given that gametophytic mutant phenotypes often show variable expression and that both *yar* and *ung9* produce aborted seeds, *yar* may well be allelic to *ung9*. However, the 3'-end of *Ds* in *yar* could not be isolated by either TAIL-PCR or iPCR, possibly because it was deleted during the transposition process. We do currently not know whether this insertion only disrupts one gene or is also flanked by an adjacent deletion. Given that *AIR9* is in the vicinity of the *Ds* launch site (DsG1; Sundaresan *et al.*, 1995), and that several deletions were created by intrachromosomal excision of a hybrid *Ds* element from this site (Page *et al.*, 2004), it is possible

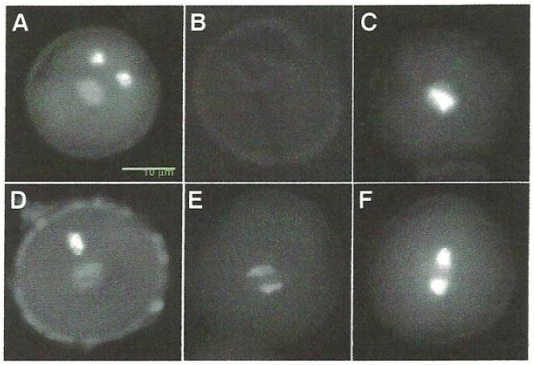


Fig. 5. Patterns of DAPI fluorescence in mature pollen grains. (A) *Wild type.* **(B-F)** *Defective pollen grains from various mutants.* **(A)** *Pollen grain containing a weakly stained, large vegetative cell and two strongly stained, small sperm cell nuclei.* **(B,C)** *Pollen grains from* amn. **(B)** *No nuclei are detectable in this pollen grain.* **(C)** *Only a single, strongly stained nucleus is present in this* amn *pollen grain.* **(D)** *Pollen grain from* aps *with only one sperm and one vegetative cell nucleus.* **(E)** *Two weakly stained, elongated and possibly degrading nuclei are visible in this* yar *pollen grain.* **(F)** *Only two strongly stained sperm-like nuclei are detected in this* did *pollen grain.*

that *ung9* and *yar* are also deletion mutants. Because of the complex findings with *Ds* as well as T-DNA insertions in *AIR9*, it is currently not possible to definitively assign the *yar* phenotype to this auxin-induced gene.

The* didilia *mutant has a gametophytic maternal post-fertilization defect

In the gametophytic maternal effect class of mutants the phenotype is apparent only at the post-fertilization stage (Grossniklaus *et al.*, 1998, Brukhin *et al.*, 2005a). Seed abortion depends on the genotype of female gametophyte only and leads to maternally derived seed abortion irrespective of the paternal contribution. The mutant *didilia (did)* was named after the Slavic goddess of female fertility, childbearing, growth and vegetation. The Kan^r:Kan^s segregation ratio of 1.05:1 was typical for a gametophytic mutation, but transmission of *did* was reduced through both the female (TEF=34%) and male gametophyte (TEM=59%) (Table 1). Mature *did* siliques contained 25% aborted seeds, which were either white in immature green siliques (Fig. 6D) or brown and shrivelled in mature siliques. 5% of the ovules remained unfertilized in the mature fruit (Fig. 6D, arrows). This seed abortion phenotype may reflect incomplete penetrance of the gametophytic mutation. Cytological observations revealed that 5% of the unfertilized ovules were arrested at the mature embryo sac stage (FG7) (not shown), while 25% of seeds arrested much later, mainly at the late heart to early torpedo stage of embryo development (Fig. 6F). The remaining 70% of seeds were normal (Fig. 6E, Fig. 1B). In arrested seeds the endosperm was also blocked at the early alveolar stage (Fig. 6F). *DID/did* mutant plants produced around 8% abnormal pollen grains (Table 2), with only two nuclei, either with one sperm-like and one vegetative nucleus or with two weakly staining, elongated nuclei. Rarely, two sperm-like, strongly stained nuclei were present (Fig. 5F).

In *did*, the first intron of *At2g01110* was disrupted by the *Ds* element (Fig. 3). This gene encodes a Twin-Arginine Translocation C (TATC) protein responsible for thylakoid membrane organization and biogenesis (Motohashi *et al.*, 2001; Allen *et al.*, 2002). TATC is a major component of ΔpH-dependent protein transporter activity (Yen *et al.*, 2002). The GUS reporter on the *Ds* element showed strong expression in both seedlings (Fig. 6H) and mature plants, including all parts of the flower (not shown). The ovules also expressed GUS (Fig. 6G) and after fertilization, we observed GUS activity in the endosperm but not the embryo (not shown). Assessment of the available expression arrays using Genevestigator tools (Hruz *et al.*, 2008) showed that *At2g01110* is expressed in many vegetative and generative organs with a particularly high activity in seeds, embryos, flowers, pedicels, and leaf primordia. During plant development gene expression peaks at the flowering stage. Mutants affecting *At2g01110* were also isolated in a screen for seedling lethality (Budziszewski *et al.*, 2001) and the protein has been detected in the proteomes of mitochondria (van der Merwe and Dubery, 2007) and chloroplasts (Zybailov *et al.*, 2008). Other mutants disrupting *At2g01110* were previously identified as *albino and pale green2* (*apg2*) (Motohashi *et al.*, 2001) and *unfertilized embryo sac3* (*une3*) (Pagnussat *et al.*, 2005). The latter shows that the gene plays an important role in zygote and endosperm formation after double fertilization (Pagnussat *et al.*, 2005). Analysis of two additional insertion lines, (GT_5_74422 and GT_5_105311) carrying an exonic *Ds* element in *At2g01110*, as well as the analysis of the *une3* mutant, revealed fertility phenotypes similar to that of *did* (Table 1S). Thus, *did* is likely allelic to *apg2* and *une3*, and the maternal *did* phenotype can be assigned to a disruption of *At2g01110*, which is expected to lead to defective plastids and/or mitochondria, which are maternally inherited.

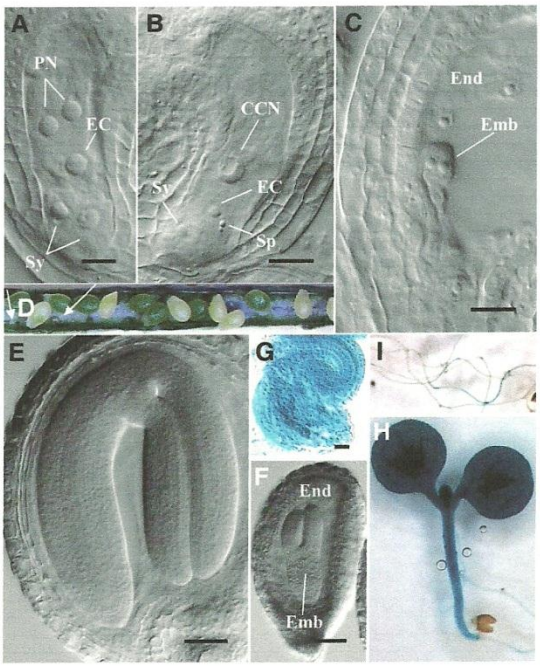


Fig. 6. Embryo sac and seed set phenotypes of the degenerative class mutant *yarilo* and the gametophytic maternal effect class *mutant didilia*. **(A-C, I)** yarilo, **(D-H)** didilia. **(A)** *Abnormally sized egg cell, synergid and polar nuclei that remain un-fused.* **(B)** *Degenerating central cell nucleus and unusually small defective egg cell nucleus. Adjacent to the egg nucleus is a smaller nucleus, probably of a sperm that has failed to fuse with the egg.* **(C)** *Shrunk degenerating two-cell embryo in the seed.* **(D)** *Siliques containing normal seed, infertile ovules (arrows), and aborting seeds (white).* **(E)** *Mature normal seed at the walking stick stage of embryo development from the same silique as shown in F.* **(F)** *Aborted seed containing arrested early torpedo stage embryo and under-developed endosperm.* **(G-I)** *GUS reporter gene expression under the promoters of the disrupted genes: The gene trap GUS gene was engineered into the* Ds, *preceded by multiple splice acceptor sites and it has no promoter, so that GUS expression can occur under the promoter of the gene disrupted by the* Ds *only when GUS inserts within a transcribed chromosomal region, creating a transcriptional fusion.* **(G)** *Cleared ovule with ubiquitous GUS expression.* **(H)** *Ubiquitous GUS expression in 10-day old seedling.* **(I)** *GUS expression in the roots of two-week old seedlings. CCN, central cell nucleus; EC, egg cell; Emb, embryo; End, endosperm; PN, polar nuclei; Sy, synergid; Sp, sperm. Bars, 10 mm in (A-C) and (G) and 50 mm in (E,F).*

Mutations reveal pleiotropic effects in gametophyte arrest

Despite of the small size and limited number of cells constituting the female gametophyte, many cellular processes are involved in its development and function (Grossniklaus and Schneitz, 1998; Yadegari and Drews, 2004; Dresselhaus, 2006). From the six classes of female gametophytic defects recognized in *Arabidopsis* (Brukhin *et al.*, 2005a), the mutants described in this paper have been assigned to four: the mitotic, karyogamic, degenerative, and maternal effect class. However, despite a prevalent stage of arrest, the phenotype was expressed at variable levels in all mutants (Fig. 1B). It is well documented in previous studies of female gametophytic mutants that developmental progression is rarely arrested at one particular stage in development (Bonhomme *et al.* 1998; Howden *et al.*, 1998; Grini *et al.*, 1999; Moore *et al.*, 1997; Pagnussat *et al.*, 2005), and that a large number of genes are expressed in the embryo sac (Yu *et al.*, 2005; Johnston *et al.*, 2007; Jones-Rhoades *et al.*, 2007; Steffen *et al.*, 2007; Wuest *et al.*, 2010). Phenotypic variability may be caused by varying degrees of carry-over of mRNA derived from earlier gene expression in the megaspore mother cell and/or by genetic redundancy in the *Arabidopsis* genome.

In addition to these difficulties, many gametophytic mutants cause maternal effects that lead to seed abortion. Pagnussat *et al.* (2005) reported that from 130 female gametophytic mutants examined, about half were found to be defective in post-fertilization processes due to the mutant maternal allele. A similar frequency was found by Moore (2002) in whose study 7 out of 14 female gametophytic mutants showed clear maternal effects. In our study, three mutants revealed a low level of seed abortion (*yar*: 3%; *amn*: 5%; *aps*: 8%) whereas *did*, which was identified as a maternal effect mutant, showed a high level of seed abortion (*did*: 25%). Late maternal effects that affect the mature seed may explain the discrepancy between the low transmission efficiency and the mild semi-sterile phenotype observed in *yar* and *aps*. In these mutants, seeds of normal appearance did not germinate or died immediately after germination (not shown).

An assessment of the defects in mature pollen grains showed that only *kup* and *yar* had a significant proportion of defective pollen (*kup*: 12%, *yar*: 23%) while the rest of the mutants produced only few abnormal pollen grains (≤10%). Except for *ast*, which has wild-type transmission through the male gametophyte, the rare phenotypes in mature pollen grains do not correlate with the strong reduction in male transmission efficiency. Therefore, there have to be defects not only during pollen development but also in the progamic phase (Lalanne *et al.*, 2004), i.e. during pollen tube growth, guidance, or reception.

Gametophytic mutants are often associated with chromosomal rearrangements

To our surprise many insertion sites turned out to be complex involving rearrangements, a feature that has often been observed for T-DNA insertions but was so far only rarely associated with *Ds* transposition. The general occurrence of *Ds*-associated deletions was estimated to be around 1% (Page *et al.*, 2004), but the current study on female gametophytic mutants, as well as a recently published study on male gametophytic mutants, in which at least 16 out of 39 *Ds* insertions were associated with rearrangements (Boavida *et al.*, 2009), show a much higher incidence of chromosomal aberrations resulting from *Ds* transposition. Because large deletions and translocations usually result in reduced gametophytic transmission, screens for gametophytic mutants may strongly enrich for chromosomal rearrangements (Oh *et al.*, 2004; Page *et al.*, 2004; Boavida *et al.*, 2009). Moreover, two of the mutants analyzed with clean *Ds* insertions (*amn* and *aps*, see Fig. 3) turned out not to be tagged by an analysis of several additional alleles. While it is known that *Ds* elements can transpose again after insertion, leaving a footprint that could cause the mutant phenotype, only few such cases have been reported for this system (Grossniklaus *et al.*, 1998; Escobar-Restrepo *et al.*, 2007). Consequently, gametophytic mutants that were isolated using *Ds* or T-DNA insertional mutagenesis have to be assessed carefully and caution has to be taken when interpreting gametophytic functions based on sequences flanking the insertion sites (Pagnussat *et al.*, 2005; Boavida *et al.*, 2009).

As a result of the aforementioned issues with *Ds*-induced mutations, only one of the six mutants described here could unambiguously be assigned to a gene: the maternal effect mutant *did*, which turned out to be allelic to *apg2* and *une3*, encoding a TACT-like protein (Motohashi *et al.*, 2001; Pagnussat *et al.*, 2005). For two other mutants, *ast* and yar, genes affected by the *Ds* insertions and the associated rearrangements are likely involved in gametophyte development, since some of the additional insertion alleles analyzed show aspects of the phenotype, but further investigations are required to unambiguously identify the gene(s) responsible for these phenotypes. Finally, *amn* and *aps* are clearly not tagged by the *Ds* despite the tight co-segregation. For *kup*, the identity of the causal gene is completely open because the two alleles available provided divergent results. Further characterization of the genes identified, the isolation of additional alleles, and the production of plants containing multiple mutations, will shed more light on the cellular functions of the biochemical pathways disrupted in the mutants presented here.

Materials and Methods

Generation of* Ds *insertion lines and plant growth conditions

We used the insertional mutagenesis system described by Sundaresan and colleagues (1995) in the wild-type strain of *Arabidopsis thaliana* (L.) Heynh. var. Landsberg *(erecta* mutant: L*er*). Mutagenesis was initiated by crossing plants homozygous for one *Ds* element to plants homozygous for an immobilized *Ac* element. Transposants were identified among the F2 seedlings on 0.7% agar medium (Difco) containing 4.4 g/L Murashige-Skoog salts (Carolina Biological) and 10 g/L sucrose (GibcoBRL) pH 5.7, 50 μg/L kanamycin A (Sigma) and 650 μg/L alpha-napthaleneacetamide (NAM) (Sigma). The T-DNAs carrying both the *Ds* element and the *Ac* element also contain the indole acetamide hydrolase (IAAH) gene. This allows selection against plants containing the IAAH gene using NAM, consequently selecting against plants containing the T-DNAs. The *Ds* element contains a kanamycin resistance (*nptII*) gene allowing plants to be recovered with a *Ds* transposon reinserted some distance from the donor locus. Insertion on a different chromosome or recombination allows the *Ds* to segregate away from the donor locus, thereby enriching for unlinked transposition events. Since this procedure also selects against the *Ac* element, the insertion is immediately stabilized. The *Ds* elements carry the β-glucuronidase (GUS) reporter gene, preceded by multiple splice acceptor sites that allow the *Ds* element to behave as a gene trap (GT) (Sundaresan *et al.*, 1995; Springer *et al.*, 1995). The reporter gene (GUS) has no promoter, so that GUS gene expression can occur under the promoter of the gene disrupted by *Ds* only when GUS inserts within a transcribed chromosomal region, creating a transcriptional fusion.

This feature permits the expression pattern of a tagged gene to be detected when the *Ds* element is inserted into a gene in the correct orientation. In practice, however, only 17% of the gene trap lines generated showed GUS-positive staining in our screen. Plants were grown on soil ED73 (Universal Erde) in a growth room with 70% relative humidity and a day–night cycle of 16 h light at 21°C and 8 h darkness at 18°C. For crosses with dehiscent anthers, closed flower buds were emasculated 1 or 2 days before pollination.

Segregation ratio distortion and semi-sterility analyses

Mutations affecting the gametophytic phase of the life cycle were identified in a two-step screen for (i) reduced fertility (seed abortion or undeveloped ovules), and (ii) segregation ratio distortion as described (Moore *et al.*, 1997; Page and Grossniklaus, 2002; Brukhin *et al.*, 2005a). Non-functional megagametophytes failed to initiate seed development resulting in semi-sterility. These mutations are marked by the *Ds* element containing a gene conferring kanamycin resistance (Kan^r) such that Kan^r is transmitted at reduced frequency to the progeny (non-Mendelian segregation). Gametophytic lethals were identified by the presence of greater than 30% undeveloped ovules in green siliques. The viable progeny was tested for segregation ratio distortion on kanamycin-containing plates. Lethality in the gametophyte or in the embryo should result in reduced transmission of Kan^r ranging from 1:1 to 2:1 kanamycin resistant to sensitive seedlings.

Genetic transmission analyses

Transmission efficiencies through male (TEM) and female (TEF) gametophytes were determined by reciprocal crosses to the wild type (Howden *et al.*, 1998; Brukhin *et al.*, 2005b). Transmission efficiency was calculated as Kan^r/Kan^s X 100%; where Kan^r, are kanamycin-resistant and Kan^s kanamycin-sensitive seedlings, respectively.

Detection of* Ds *number per gene trap (GT) transposant

We assessed the number of *Ds* elements present in each line by the genomic Southern blotting technique prior to any genetic and cytological analyses. This was necessary to ensure that a mutant phenotype was generated by a single *Ds* insertion. Genomic DNA was extracted from the inflorescence of the candidate GT line. A homogenizer (Silamat S5, Vivadent) was used to grind the frozen tissue in the presence of 4 glass beads of 200-300 micron diameter (Sigma). The resultant powder was processed using the Nucleon Phytopure Kit (Amersham) according to the standard protocol. 5 µg of genomic DNA was digested with *Eco*R1 endonuclease (Boehringer Mannheim) and the resultant fragments resolved on a 0.8% agarose gel. The fractionated fragments were then transferred to a nylon membrane (Boehringer Mannheim) using the capillary blotting method. A *Ds*-dig probe was produced by PCR amplification to allow the incorporation of digoxygenin-11-dUTP (Boehringer Mannheim) into the DNA probe. To generate the *Ds*-dig probe, primers FMI32361 (5'- ATCCCGTACCGACCGTTATCG-3') and FMI32360 (5'-CGTGTGAATGTGTGATGC-3') were used for the dig-labelling reaction (Roche Applied Science) amplifying the 5' end of *Ds* from plasmid pWS31 (Sundaresan *et al.*, 1995). Hybridization with the *Ds*-dig probe was performed overnight at 68°C, followed by membrane washes performed at 2x5 min intervals at 68°C in W1 buffer (2x SSC, 0.1% SDS); 1x15 min in W2 (0.2x SSC,0.1% SDS), and W3 (0.1x SSC, 0.1% SDS). The probe was detected using the standard protocol for chemi-luminescence with the CPD-star substrate (Boehringer Mannheim). A banding pattern was visualized by exposure of the probed nylon membrane to a standard laboratory X-Ray film (Kodak).

Identification of sequences flanking the* Ds *insertions

Chromosomal sequences flanking the gene trap insertions were amplified by TAIL-PCR (thermal asymmetric interlaced PCR) according to Liu *et al.* (1995) with modifications described by Grossniklaus and colleagues (1998), using nested primers within the *Ds* borders (*Ds3'*-1: 5'-ACC CGA CCG GAT CGT ATC GGT-3', *Ds3'*-2: 5'- CCG GTA TAT CCC GTT TTC G-3', *Ds3'*-3: 5'-GTT ACC GAC CGT TTT CAT CC-3, *Ds5'*-1: 5'-CCG TTT ACC GTT TTG TAT ATC CCG-3', *Ds5'*-2: 5'-CGT TCC GTT TTC GTT TTT TAC C-3', *Ds5'*-3: 5'-GGT CGG TAC GGA ATT CTC CC-3') and a degenerate primer (AD2: 5'-NGT CGA (G/C)(A/T)G ANA (A/T)GA A-3'). TAIL-PCR products were purified using the QiaQuick kit (Qiagen), and then sequenced. For inverse PCR (iPCR), DNA was digested with *Bst*YI for the identification of 5' end and *Nco*I for the 3'-end. Primers for detection of the 5'-end were as follows: B34: 5'-ACG GTC GGT ACG GGA TTT TCC CAT-3' (primary iPCR), 4447: 5'-CCG TTT CCG TTC CGT TTT C-3' (nested iPCR), B35: 5'-TAT CGT ATA ACC GAT TTT GTT AGT TTT ATC-3' (primary and nested), and for 3'end: pr3'end A1: 5'-CGA TTT CGA CTT TAA CCC GAC CGG AT-3' (primary), pr3'end A2: 5'-TCG TTT TCG TTA CCG GTA TAT CCC GT-3' (nested), pr3'B: 5'-GAC GGG AAC CGG TAT TTT TGT TCG GT-3' (primary and nested). Disrupted genes were physically mapped and aligned with the genomic sequence of *Arabidopsis* using the BLAST search algorithm (www.*Arabidopsis*.org). We examined the expression of the disrupted genes by Meta-Profile analysis of Genevestigator software (https://www.genevestigator.ethz.ch/gv/index.jsp), which summarizes information accumulated from hundreds of microarray experiments (Hurz *et al.*, 2008).

Insertion lines from other collections

SALK lines were ordered from the Salk Institute Genomic Analysis Laboratory La Jolla, California, through the Nottingham *Arabidopsis* Stock Centre NASC. Lines GT.100129, GT_5_48498, GT_5_74422, GT_5_105311, GT.100129 and SM_3.27652 were kindly obtained from the John Innes Centre, UK. Line *une3* was kindly provided by Prof. V. Sundaresan, (University of California, Davis). Most of the insertion SALK and GT lines showing a reduced seed set phenotype related to the corresponding mutant were genotyped in order to confirm the disruption of the gene under study.

Cytological analyses and image processing

Morphological characterization was performed on ovules and seeds cleared with chloral hydrate following the protocol of Yadegari and colleagues (1994). Specimens were observed using a Leica DMR microscope (Leica Microsystems) under differential interference contrast (DIC) optics. For quantitative phenotypic analyses of embryo sac development under DIC optics, 80 to 140 ovules, two to five days after self-pollination, were scored for each mutant in order to obtain a representative average terminal phenotype. For DAPI staining flowers were kept in 70% ethanol followed by substitution with staining buffer: CyStain UV Precise P (Partec GmbH). DAPI fluorescence in pollen grains were visualized under the microscope Leica DM 6000. All images were processed by Adobe Photoshop 5.5 (Adobe Systems).

Acknowledgements

We thank Juan-Miguel Escobar-Restrepo (University of Zürich) for help with the interpretation of sequencing data. This work was supported by the University of Zürich, the EXOTIC project of Framework Program V of the European Union, the Novartis Foundation, and grants of the Swiss National Science Foundation to UG. VB is grateful to the Kredit zur Förderung des akademischen Nachwuchses der Stiefel-Zangger-Stiftung for financial support. We wish to express our gratitude to Dr. Caroline Ford (Aberystwyth University) for the critical reading of this manuscript.

References

ALLEN, S.C., BARRETTt, C.M., RAY, N., ROBINSON, C. (2002). Essential cytoplasmic domains in the *Escherichia coli* TatC protein. *J. Biol. Chem.* 277: 10362-10366.

BAYER, E. M., BOTTRILL, A. R., WALSHAW, J., VIGOROUX, M., NALDRETT, M. J., THOMAS, C.L., MAULE, A.J. (2006). *Arabidopsis* cell wall proteome defined

using multidimensional protein identification technology. *Plant Proteomics* 6: 301-311.

BERNAL A.J., YOO C.M., MUTWIL M., JENSEN J.K., HOU G., BLAUKOPF C., SORENSEN I., BLANCAFLOR E.B., SCHELLER H.V., WILLATS W.G. (2008). Functional analysis of the cellulose synthase like genes *CSLD1*, *CSLD2* and *CSLD4* in tip growing *Arabidopsis* cells. *Plant Physiology,* 148: 1238-1253.

BOAVIDA, L.C., SHUAI, B., YU, H.J., PAGNUSSAT, G.C., SUNDARESAN, V., MCCORMICK, S. (2009). A collection of *Ds* insertional mutants associated with defects in male gametophyte development and function in *Arabidopsis thaliana*. *Genetics,* 181: 1369-1385.

BONHOMME, S., HORLOW, C., VEZON, D., DE LAISSARDIERE, S., GUYON A., *et al.* (1998). T-DNA mediated disruption of essential gametophytic genes in *Arabidopsis* is unexpectedly rare and cannot be inferred from segregation distortion alone. *Mol.Gen. Genet.* 260: 444–452.

BRUKHIN, V., CURTIS, M.D., GROSSNIKLAUS, U. (2005a). The angiosperm female gametophyte: no longer the forgotten generation. *Current Science* 89: 1844-1852.

BRUKHIN, V., GHEYSELINCK, J., GAGLIARDINI, V., GENSCHIK, P., GROSSNIKLAUS, U. (2005b). The RPN1 subunit of the 26S proteasome in *Arabidopsis* is essential for embryogenesis. *Plant Cell* 17: 2723-2737.

BUDZISZEWSKI, G.J., LEWIS, S.P., GLOVER, L.W., REINEKE, J., JONES, G., *et al.* (2001). *Arabidopsis* genes essential for seedling viability: isolation of insertional mutants and molecular cloning. *Genetics* 159: 1765-1778.

BUSCHMANN, H., CHAN, J., SANCHEZ-PULIDO, L., ANDRADE-NAVARRO, M.A., DOONAN, J.H., LLOYD, C.W. (2006). Microtubule-associated AIR9 recognizes the cortical division site at preprophase and cell-plate insertion. *Curr. Biol.* 16: 1938-1943.

CHRISTENSEN, C. A., KING, E.J., JORDAN, J.R., DREWS, G.N. (1997). Megagametogenesis in *Arabidopsis* wild type and the *Gf* mutant. *Sex. Plant Reprod.* 10: 49–64.

CHRISTENSEN, C. A., SUBRAMANIAN, S., DREWS, G. N. (1998). Identification of gametophytic mutations affecting female gametophyte development in *Arabidopsis*. *Dev. Biol.* 202: 136–151.

CHRISTENSEN, C.A., GORSICH, S.W., BROWN, R.H., JONES, L.G., BROWN, J., SHAW, J.M., DREWS, G.N. (2002). Mitochondrial GFA2 is required for synergid cell death in *Arabidopsis*. *Plant Cell* 14: 2215-2232.

DEN ELZEN, N., PINES, J. (2001). Cyclin a is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J. Cell Biol.* 153: 121-135.

DRESSELHAUS T. 2006 Cell-cell communication during double fertilization. *Curr Opin Plant Biol.* 9: 41-47.

DREWS, G. N., LEE, D., CHRISTENSEN, C.A. (1998). Genetic analysis of female gametophyte development and function. *Plant Cell* 10: 5-17.

ESCOBAR-RESTREPO, J.M., HUCK, N., KESSLER, S., GAGLIARDINI, V., GHEYSELINCK, J., YANG, W.C., GROSSNIKLAUS, U. (2007). The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. *Science* 317: 656-860.

FELDMANN, K. A., COURY, D. A., CHISTIANSON, M.L. (1997). Exceptional segregation of a selectable marker (KanR) in *Arabidopsis* identifies genes important for gametophytic growth and development. *Genetics* 147: 1411-22.

GALLOIS, J.L., GUYON-DEBAST, A., LÉCUREUIL, A., VEZON, D., CARPENTIER, V., BONHOMME, S., GUERCHE, P. (2009). The *Arabidopsis* proteasome RPT5 subunits are essential for gametophyte development and show accession-dependent redundancy. *The Plant Cell,* 21(2): 442-459.

GRINI, P. E., SCHNITTGER, A., SCHWARZ, H., ZIMMERMANN, I., SCHWAB, B., *et al.* (1999). Isolation of ethyl methanesulfonate-induced gametophytic mutants in *Arabidopsis thaliana* by a segregation distortion assay using the multimarker chromosome 1. *Genetics* 151: 849-863.

GROSSNIKLAUS, U., SCHNEITZ, K. (1998). The molecular and genetic basis of ovule and megagametophyte development. *Seminars in Cell & Developmental Biology* 9: 227-238.

GROSSNIKLAUS, U., VIELLE-CALZADA, J. P., HOEPPNER, M. A., GAGLIANO, W. B. (1998). Maternal control of embryogenesis by *MEDEA*, a *polycomb* group gene in *Arabidopsis*. *Science* 280: 446–450.

HESLOP-HARRISON, J. (1979). The forgotten generation: some thoughts on the genetics and physiology of angiosperm gametophytes. *The Bateson Lecture: Proceedings of the Fourth John Innes Symposium*, pp. 1–14.

HOWDEN, R., S., PARK, K., MOORE, J. M., ORME, J., GROSSNIKLAUS, U. *et al.*,1998). Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in *Arabidopsis*. *Genetics* 149: 621–631.

HRUZ, T., LAULE, O., SZABO, G., WESSENDORP, F., BLEULER, S., OERTLE, L., WIDMAYER, P., GRUISSEM, W., ZIMMERMANN, P. (2008). Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformat* 2008: 1-5.

JONES-RHOADES, M.W,, BOREVITZ, J.O,, PREUSS, D. (2007). Genome-wide expression profiling of the *Arabidopsis* female gametophyte identifies families of small, secreted proteins. *PLoS Genet.* 3: 1848-1861.

JOHNSTON, A.J., MEIER, P., GHEYSELINCK, J., WUEST, S.E., FEDERER, M., SCHLAGENHAUF, E., BECKER, J.D., GROSSNIKLAUS, U. (2007). Genetic subtraction profiling identifies genes essential for *Arabidopsis* reproduction and reveals interaction between the female gametophyte and the maternal sporophyte. *Genome Biol* 8: 204-209.

KIPREOS E.T., PAGANO, M. 2000. The F-box protein family. *Genome Biol.* 1 (5), 3002.

LALANNE, E., MICHAELIDIS, C., MOORE, J.M., GAGLIANO, W., JOHNSON, A. *et al.* (2004). Analysis of transposon insertion mutants highlights the diversity of mechanisms underlying male progamic development in *Arabidopsis*. *Genetics* 167: 1975-1986.

LIU, Y. G., MITSUKAWA, N., OOSUMI, T., WHITTIER, R. F. (1995). Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J.* 8: 457–463.

MOORE, J. M., VIELLE CALZADA, J.-P., GAGLIANO, W., GROSSNIKLAUS, U. (1997). Genetic characterization of *hadad*, a mutant disrupting female gametogenesis in *Arabidopsis thaliana*. *Cold Spring Harbor Symp. Quant. Biol.* 62: 35-47.

MOORE, J. M. (2002). *Isolation and characterization of gametophytic mutants in Arabidopsis thaliana. Ph.D. Thesis*, State University of New York at Stony Brook, USA.

MOTOHASHI, R., NAGATA, N., ITO, T., TAKAHASHI, S., HOBO, T. *et al.* (2001). An essential role of a TatC homologue of a Delta pH-dependent protein transporter in thylakoid membrane formation during chloroplast development in *Arabidopsis Thaliana*. *Proc Natl Acad Sci USA* 98: 10499-10504.

NEUTEBOOM, L. W., NG, J. M., KUYPER, M., CLIJDESDALE, O. R., HOOYKAAS, P. J., *et al.* (1999). Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation. *Plant Mol. Biol.* 39: 273–287.

OH S-A, PARK S-K, JANG I, MOORE JM, GROSSNIKLAUS U, TWELL D. (2003). *halfman1*, an *Arabidopsis* male gametophytic mutant associated with a 150 kb chromosomal deletion at the site of transposon insertion. *Sex. Plant Reprod.* 16: 99-102.

OLSEN, O.A. (2004). Nuclear endosperm development in cereals and *Arabidopsis thaliana*. *Plant Cell*, 16: 214-227.

PAGE, D.R., GROSSNIKLAUS, U. (2002). The art and design of genetic screens: *Arabidopsis thaliana*. *Nat. Rev. Genet.* 3: 124-136.

PAGE, D.R., KÖHLER, C., DA COSTA-NUNES, J.A., BAROUX, C., MOORE, J.M., GROSSNIKLAUS, U. (2004). Intrachromosomal excision of a hybrid *Ds* element induces large genomic deletions in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 101: 2969-2974.

PAGNUSSAT, G.C., YU, H.J., NGO, Q.A., RAJANI, S., MAYALAGU, S. *et al.* 2005. Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. *Development* 132: 603-614.

PARINOV, S., SEVUGAN, M., YE, D., YANG, W.-C., KUMARAN, M., SUNDARESAN, V. (1999). Analysis of flanking sequences from *Dissociation* insertion lines: a database for reverse genetics in *Arabidopsis*. *Plant Cell* 11: 2263-2270.

REDEI, G. P. (1965). Non-Mendelian megagametogenesis in *Arabidopsis*. *Genetics*, 51: 857–872.

SMALLE, J., VIERSTRA, R.D. (2004). The ubiquitin 26S proteasome protealitic pathway. Annu. Rev. Plant Physiol. *Plant Mol. Biol.* 55: 555–590.

SPRINGER, P. S., MCCOMBIE, W. R., SUNDARESAN, V., MARTIENSSEN, R. A. (1995). Gene trap tagging of *prolifera*, an essential *Mcm2-3-5*-like gene in *Arabidopsis*. *Science*, 268: 877–880.

STEFFEN, J.G., KANG, I.-H., MACFARLANE, J., DREWS, G.N. (2007). Identification of genes expressed in the *Arabidopsis* female gametophyte. *Plant J.* 5: 1281-1292.

SUNDARESAN, V., SPRINGER, P., VOLPE, T., HAWARD, S., JONES, J. D. *et al.* (1995). Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements. *Genes Dev.* 9: 1797–1810.

TAKAHASHI, H., WATANABE-TAKAHASHI, A., SMITH, F. W., BLAKE-KALFF, M., HAWKESFORD, M. J., SAITO, K. (2000). The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. *Plant J.* 23: 171-182.

VAN DER MERWE, J.A., DUBERY, I.A. (2007). Expression of mitochondrial tatC in *Nicotiana tabacum* is responsive to benzothiadiazole and salicylic acid. *J. Plant Physiol.* 164: 1231-1234.

VAN ENCKEVORT, L.J., DROC, G., PIFFANELLI, P., GRECO, R., GAGNEUR, C. (2005). EU-OSTID: a collection of transposon insertional mutants for functional genomics in rice. *Plant Mol. Biol.* 59: 99-110.

VIERSTRA, R.D. (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends Plant Sci.* 68: 135–142.

WUEST S.E., VIJVERBERG K., SCHMIDT A, WEISS M., GHEYSELINCK J., LOHR M., WELLMER F., RAHNENFÜHRER J., VON MERING C., GROSSNIKLAUS U. (2010). *Arabidopsis* female gametophyte gene expression map reveals similarities between plant and animal gametes. *Curr Biol.* 20: 506-512.

YADEGARI, R., PAIVA, G., LAUX, T., KOLTUNOW, A.M., APUYA, N., *et al.* (1994). Cell differentiation and morphogenesis are uncoupled in *Arabidopsis raspberry* embryos. *Plant Cell* 6: 1713-1729.

YADEGARI, R., DREWS, G.N. (2004). Female gametophyte development. *Plant Cell* 16: 133-141.

YANG, P., FU, H., WALKER, J., PAPA, C.M., SMALLE, J., *et al.* (2004). Purification of the *Arabidopsis* 26–S proteasome: biochemical and molecular analyses revealed the presence of multiple isoforms. *J. Biol. Chem.* 279: 6401-6413.

YEN, M. R., TSENG, Y. H., NGUYEN, E. H., WU, L. F., JR SAIER, M. H. (2002). Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Arch. of Microbiol.* 177: 441-450.

YU, H.J., HOGAN, P., SUNDARESAN, V. (2005). Analysis of the female gametophyte transcriptome of *Arabidopsis* by comparative expression profiling. *Plant Physiol.* 139: 1853-1869.

ZHANG, S., RAINA, S., LI, H., LI, J., DEC, E., MA, H., HUANG, H., FEDEROFF, N.V. (2003). Resources for targeted and deletional mutagenesis in *Arabidopsis*. *Plant Molec. Biol.* 53: 133-150.

ZYBAILOV, B., RUTSCHOW, H., FRISO, G., RUDELLA, A., EMANUELSSON, O., SUN, Q., VAN WIJK, K. J. (2008). Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE* 3(4): 1994.

The Arabidopsis CUL4-DDB1 Complex Interacts with MSI1 and is Required to Maintain *MEADEA* Parental Imprinting

The EMBO Journal (2011) 30, 731–743 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
www.embojournal.org

THE EMBO JOURNAL

The Arabidopsis CUL4–DDB1 complex interacts with MSI1 and is required to maintain *MEDEA* parental imprinting

Eva Dumbliauskas[1], Esther Lechner[1], Miłosława Jaciubek[2], Alexandre Berr[1], Maghsoud Pazhouhandeh[1], Malek Alioua[1], Valerie Cognat[1], Vladimir Brukhin[2,4], Csaba Koncz[3], Ueli Grossniklaus[2], Jean Molinier[1] and Pascal Genschik[1,*]

[1]Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Unité Propre de Recherche, Conventionné avec l'Université de Strasbourg, Strasbourg, France, [2]Institute of Plant Biology and Zürich-Basel Plant Science Center, University of Zürich, Zollikerstrasse, Zürich, Switzerland and [3]Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg, Köln, Germany

Protein ubiquitylation regulates a broad variety of biological processes in all eukaryotes. Recent work identified a novel class of cullin-containing ubiquitin ligases (E3s) composed of CUL4, DDB1, and one WD40 protein, believed to act as a substrate receptor. Strikingly, CUL4-based E3 ligases (CRL4s) have important functions at the chromatin level, including responses to DNA damage in metazoans and plants and, in fission yeast, in heterochromatin silencing. Among putative CRL4 receptors we identified MULTICOPY SUPPRESSOR OF IRA1 (MSI1), which belongs to an evolutionary conserved protein family. MSI1-like proteins contribute to different protein complexes, including the epigenetic regulatory *Polycomb* repressive complex 2 (PRC2). Here, we provide evidence that Arabidopsis MSI1 physically interacts with DDB1A and is part of a multimeric protein complex including CUL4. CUL4 and DDB1 loss-of-function lead to embryo lethality. Interestingly, as in *fis* class mutants, *cul4* mutants exhibit autonomous endosperm initiation and loss of parental imprinting of *MEDEA*, a target gene of the Arabidopsis PRC2 complex. In addition, after pollination both *MEDEA* transcript and protein accumulate in a *cul4* mutant background. Overall, our work provides the first evidence of a physical and functional link between a CRL4 E3 ligase and a PRC2 complex, thus indicating a novel role of ubiquitylation in the repression of gene expression.

The EMBO Journal (2011) **30,** 731–743. doi:10.1038/emboj.2010.359; Published online 14 January 2011
Subject Categories: chromatin & transcription; plant biology
Keywords: Arabidopsis; *CUL4*; parental imprinting; *Polycomb* repressive complex 2; seed development

*Corresponding author. Institut de Biologie Moléculaire des Plantes (CNRS), 12, rue du Général Zimmer 67084 Strasbourg, France. Tel.: +33 3 88 41 72 78; Fax: +33 3 88 61 44 42; E-mail: pascal.genschik@ibmp-cnrs.unistra.fr
[4]Present address: Aberystwyth University, IBERS, Edward Llwyd Building, Penglais, Aberystwyth, Ceredigion SY23 3DA, UK

Received: 6 November 2010; accepted: 13 December 2010; published online: 14 January 2011

Introduction

Regulation of protein stability by the ubiquitin/proteasome system participates in a broad variety of physiologically and developmentally controlled processes in all eukaryotes (Ciechanover *et al*, 2000; Smalle and Vierstra, 2004). In this pathway, a critical step involves ubiquitin ligases (E3s), which facilitate the transfer of ubiquitin moieties to a substrate protein, the preparative step for degradation via the 26S proteasome. Among the different E3 enzymes, the composition of CUL4-based E3 ligases (CRL4s) was only recently identified (Higa and Zhang, 2007). CUL4 binds RBX1 to recruit a specific E2 ubiquitin-conjugating enzyme, and also binds DDB1, an adaptor protein, which itself associates with a substrate receptor. Affinity purification of CLR4s from mammalian cells identified various WD40 proteins as possible substrate receptors (Angers *et al*, 2006; He *et al*, 2006; Higa *et al*, 2006; Jin *et al*, 2006). Many of these proteins, also called DDB1 and CUL4-associated factors (DCAFs), contain WDxR motifs that are required for efficient DDB1 binding. However, for most of them, their roles and substrates remain unknown. In humans, about 90 different DCAFs have been predicted (He *et al*, 2006), suggesting the existence of a large number of CRL4s. A similar number of WD40 repeat proteins harbouring at least one WDxR motif have been identified in the model plant *Arabidopsis thaliana* (Lee *et al*, 2008). One of the predicted Arabidopsis DCAFs is MULTICOPY SUPPRESSOR OF IRA1 (MSI1), which belongs to an evolutionary conserved protein family (reviewed in Hennig *et al*, 2005), whose founding member is MSI1 from yeast (Ruggieri *et al*, 1989). In both metazoans and plants, MSI1-like proteins are part of several protein complexes involved in diverse chromatin functions (reviewed in Hennig *et al*, 2005). In particular, MSI1 has been proposed to maintain epigenetic memory during development by targeting silencing complexes to chromatin.

In Arabidopsis, MSI1 is essential for plant reproductive development (Köhler *et al*, 2003; Guitton *et al*, 2004). In *msi1* mutants, seeds abort when the mutant allele is inherited from the mother regardless of the paternal contribution. In such seeds, the endosperm (an embryo nourishing tissue) does not cellularize, whereas the embryo exhibits cell-cycle and developmental defects. *msi1* mutants have a strong penetrance of autonomous endosperm development in the absence of fertilization and form rare parthenogenetic embryos (Köhler *et al*, 2003; Guitton and Berger, 2005). MSI1 is part of the FIS–PRC2 complex together with at least three other proteins, MEDEA (MEA), FERTILIZATION-INDEPENDENT SEED2 (FIS2) and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE), which is required for normal seed development (Köhler *et al*, 2003). *MEA* encodes a SET-domain-containing histone methyltransferase homologous to *Drosophila* Enhancer of Zeste (Grossniklaus *et al*, 1998) and regulates the imprinted expression of itself, as well as of its target gene *PHERES1*

(*PHE1*), encoding a MADS-domain transcription factor (Köhler *et al*, 2005). Imprinting regulation by FIS–PRC2 involves the silencing of the paternal allele of *MEA* and the maternal allele of *PHE1*, respectively (Köhler *et al*, 2005; Baroux *et al*, 2006; Gehring *et al*, 2006; Jullien *et al*, 2006). In contrast, auto-repression of the maternal *MEA* allele is FIS–PRC2 independent (Baroux *et al*, 2006).

Here, we report that all WD40 repeat MSI1-like proteins from various organisms carry at least one conserved WDxR motif, a signature of DCAFs. Arabidopsis MSI1 physically interacts with DDB1A and is part of a CUL4–DDB1A–MSI1 protein complex. Functional analysis revealed that *CUL4*, as well as the Arabidopsis *DDB1* homologs, are essential for seed production. Importantly, the *cul4* mutation leads to autonomous endosperm development and loss of parental *MEA* imprinting, that is reactivation of the paternal *MEA* allele, supporting a functional link of this E3 ligase and the FIS–PRC2 complex.

Results

MSI-like proteins are evolutionary conserved WD40 proteins that carry WDxR motifs

Recent work identified DDB1 and DCAFs as possible substrate receptors of CRL4 E3 ligases (reviewed in Lee and Zhou, 2007). The largest class of DCAFs are WD40 repeat proteins, which interact with DDB1 via one or several conserved WDxR motifs. The Arabidopsis genome encodes 237 WD40 repeat proteins; however, only a subset of them (~80 proteins) carry one or more WDxR motif(s) (Lee *et al*, 2008 and our unpublished data). Among these proteins we identified MSI1 and four other Arabidopsis MSI1-related proteins, named MSI2–MSI5 (reviewed in Hennig *et al*, 2005). When all MSI1-like proteins from plant and non-plant organisms were compared, it appeared that most of them share a highly conserved WDxR motif (Figure 1). In metazoans, MSI1-like proteins exhibit also a second WDxR motif, which is less conserved in plants, but is also present in fungi. Therefore, most if not all MSI1-like proteins are structurally related to DCAFs.

MSI1 associates with DDB1A and CUL4 in Arabidopsis

We first investigated whether MSI1 interacts with DDB1A in a yeast two-hybrid assay. Similarly to DDB2 (Molinier *et al*, 2008), which served as a positive control, MSI1 and DDB1A interacted, although the interaction was weak as yeast growth was only detected on (-LWH) medium (Figure 2A). We further confirmed this interaction by an *in vitro* pull-down assay. In this experiment, a fusion protein between glutathione-S-transferase (GST) and DDB1A, GST-DDB1A, was incubated with *in vitro* translated, ^{35}S-methionine-labelled MSI1 or DDB2. Consistently, MSI1 and DDB2 co-precipitated with GST-DDB1A, but not with GST alone (Figure 2B). To provide evidence for a physical interaction between both proteins in plant cells, we carried out bimolecular fluorescence complementation (BiFC) experiments. Plasmids YC-MSI1 and YN-DDB1A were co-bombarded into etiolated mustard hypocotyls. A strong YFP signal was observed in the nucleus of 81% examined cells (35/43; Figure 2C). These data are similar to those obtained with cells transformed with the positive control YN-DDB1A + YC-DDB2 (43/46). Only a weak fluorescence signal was observed after bombardment with the following plasmid combinations YN-DDB1A + YC-BPM3 (9/35) and YN-BPM3 + YC-MSI1 (2/27), where BPM3 (BTB/POZ-MATH3 protein encoded by *At2g39760*) is a nuclear cullin-ring ubiquitin ligase3 (CLR3) receptor, used here as a negative control. Taken together, our data clearly demonstrate a physical interaction between DDB1A and MSI1.

Next, we tested whether MSI1 is also part of a protein complex containing Arabidopsis CUL4. Thus, we immunoprecipitated Arabidopsis CUL4 from plants expressing the MSI1–RFP fusion protein under the control of its own promoter (Chen *et al*, 2008). Hence, MSI1 was successfully co-immunoprecipitated in this assay (Figure 2D). Since CUL4 interacts with DDB1A (Bernhardt *et al*, 2006) our results, collectively, support the existence of a CUL4–DDB1A–MSI1 protein complex in Arabidopsis.

CUL4 and its adaptors DDB1A and DDB1B are required for embryogenesis

In Arabidopsis, loss-of-function of *MSI1* causes maternal effect embryo lethality leading to seed abortion early in development (Köhler *et al*, 2003). We have previously isolated a T-DNA mutant, *cul4-1* (Bernhardt *et al*, 2006), in which *CUL4* expression was severely downregulated. Although viable *cul4-1* homozygous mutants were obtained, these plants showed various developmental abnormalities (Bernhardt *et al*, 2006). When selfed, we noticed that *cul4-1* homozygous plants exhibited altered seed development leading eventually to seed abortion (Supplementary Figure S1). Thus, we examined *cul4-1* homozygous mutant seeds at different developmental stages (Figure 3). Already at the octant stage, we observed a lower proliferation of the endosperm (Figure 3B) while at later seed developmental stages we scored abnormally large endosperm nuclei and delayed embryo development (Figure 3D and F). Because of the pleiotropic and hypomorphic nature of the *cul4-1* allele, we aimed to identify amorphic *CUL4* loss-of-function mutants. As no such mutants were available in public collections, we screened a collection of Arabidopsis T-DNA insertion lines (Ríos *et al*, 2002). Two T-DNA insertions were identified within the coding region of *CUL4*, called *cul4-2* and *cul4-3* (Supplementary Figure S2A). Both *cul4-2* and *cul4-3* mutants were backcrossed to the wild type and Southern blots confirmed single T-DNA insertions. Although we genotyped 137 and 72 progeny from selfed *cul4-2* and *cul4-3* mutant plants, respectively, we were unable to identify homozygous mutants, suggesting that *CUL4* is an essential gene in Arabidopsis.

As both lines contained single T-DNA insertions with integral hygromycin selection markers, we self-pollinated *cul4-2* and *cul4-3* heterozygous plants and analysed the segregation of this marker among their progeny (Table I). This genetic analysis revealed a segregation ratio close to 2:1 consistent with nearly fully penetrant zygotic embryo lethality. Because the segregation ratio of the marker was slightly below 2:1 for the *cul4-2* allele, suggesting a weak defect in gametophytic transmission, we performed reciprocal crosses with wild-type plants. The transmission efficiency of the marker was slightly reduced through both male and female gametophytes (Table I).

Next, we examined mature siliques for the presence of aborted seeds. The number of aborted seeds was consistent with zygotic embryo lethality, where a segregation

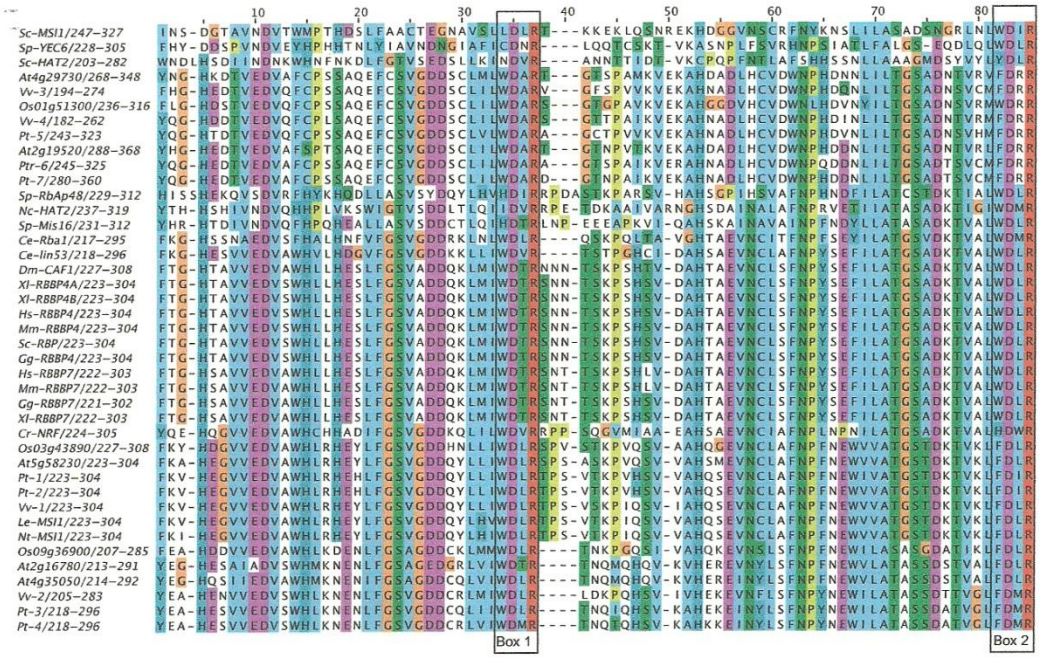


Figure 1 Alignments of MSI1-like proteins and WDxR motifs. All five Arabidopsis MSI1-like protein sequences (MSI1, AT5G58230; MSI2, AT2G16780; MSI3, AT4G35050; MSI4, AT2G19520 and MSI5, AT4G29730) were used to identify MSI1-like proteins by BLAST (Altschul *et al*, 1990). We used the following databases: for *Oryza sativa* (http://rice.plantbiology.msu.edu/; Os03g43890; Os09g36900; Os01g51300); *Vitis vinifera* (http://www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html; Vv-1 GSVIVP00030810001; Vv-2 GSVIVP00036121001; Vv-3 GSVIVP00016560001; Vv-4 GSVIVP00034167001); *Lycopersicon esculentum* (NCBI; http://www.ncbi.nlm.nih.gov/; Le-MSI1 O22466.1); *Nicotiana tabacum* (NCBI; Nt-MSI1 ABY84675.1); *Homo sapiens* (NCBI; Hs-RBBP4 NP_005601.1; Hs-RBBP7 NP_002884.1); *Mus musculus* (NCBI; Mm-RBBP4 NP_033056.2; Mm-RBBP7 NP_033057.3); *Gallus gallus* (NCBI; Gg-RBBP4 Q9W7I5.3; Gg-RBBP7 Q9I8G9.1); *Drosophila melanogaster* (NCBI; Dm-CAF-1 NP_524354.1); *Caenorhabditis elegans* (NCBI; Ce-lin53 NP_492552.1; Ce-Rba1 NP_492551.1); *Xenopus laevis* (NCBI; Xl-RBBP4B Q6INH0.3; Xl-RBBP4A O93377.3; Xl-RBBP7 Q8AVH1.1); *Chlamydomonas reinhardtii* (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html; Cr-NRF XP_001696907.1); *Populus trichocarpa* (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Pt-1 estExt_fgenesh4_pg.C_LG_II1945; Pt-2 estExt_fgenesh4_pg.C_LG_XIV1179; Pt-3 gw1.IX.1159.1; Pt-4 estExt_fgenesh4_pg.C_LG_IV1464; Pt-5 eugene3.00440093; Ptr-6 gw1.145.113.1; Pt-7 eugene3.02850001); *Schizosaccharomyces pombe* (NCBI; Sp-RbAp48 O14021.1; Sp-YEC6 Q9Y825.1; Sp-Mis16 NP_587881.1); *Saccharomyces cerevisiae* (NCBI; Sc-HAT2 P39984.1; Sc-MSI1 P13712.1; Sc-RBP 1919423A), and *Neurospora crassa* (NCBI; Nc-HAT2 Q7S7N3.2). All proteins identified were aligned using the program Muscle v3.6 (Edgar, 2004). Non-conserved protein regions were removed by GBlocks v0.91b using the following settings: minimum number of sequences for a conserved position: 21; minimum number of sequences for a flanking position: 34; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 5; allowed gap positions: with half. The positions of two conserved WDxR motifs are indicated (Box1 and Box2).

of aborted:normal seeds of 1:3 is expected (Table II; Supplementary Figure S1). To further investigate at which developmental stage embryogenesis is arrested, we analysed cleared seed specimens from siliques of selfed *cul4-2* mutant plants at different developmental stages. At the octant stage, the mutant seeds exhibited a low number of large nuclei in the endosperm (Figure 3H). At later stages, embryos arrested their development at the globular stage with abnormal shapes and cell division defects in both the suspensor and the embryo proper (Figure 3J and L). Moreover, in *cul4-2* homozygous mutant seeds, the endosperm was always severely underdeveloped with a dozen fewer enlarged, abnormal nuclei. When siliques were analysed at later stages, harbouring bent-cotyledon stage or mature wild-type sibling embryos, the arrested seeds had degenerated (not shown), indicating a strict arrest and not only a delay in seed development. Similar results were obtained with the *cul4-3* mutant allele (Supplementary Figure S3B).

Because CUL4 interacts with DDB1 to form CRL4 E3 complexes, we also investigated whether DDB1 is required for embryogenesis. The Arabidopsis genome encodes two expressed DDB1-related proteins, named *DDB1A* (*At4g05420*) and *DDB1B* (*At4g21100*), exhibiting 89% sequence identity at the amino-acid level (Schroeder *et al*, 2002). DDB1A loss-of-function mutants are viable (Molinier *et al*, 2008). Therefore, we searched for T-DNA insertion mutants in the related *DDB1B* gene and identified one mutant, named *ddb1b-1*, from the SALK collection (SALK 061944) (Alonso *et al*, 2003). In the *dbb1b-1* allele, the T-DNA interrupts the coding sequence in the last exon (Supplementary Figure S2B). Homozygous *ddb1b-1* mutant plants developed normally and were fully fertile. To test whether *DDB1A* and *DDB1B* act redundantly during embryogenesis, the *ddb1a-2* mutant was used to pollinate a homozygous *ddb1b-1* mutant plant. Among the progeny of this cross, we selected F2 plants that were *DDB1A/ddb1a-2*

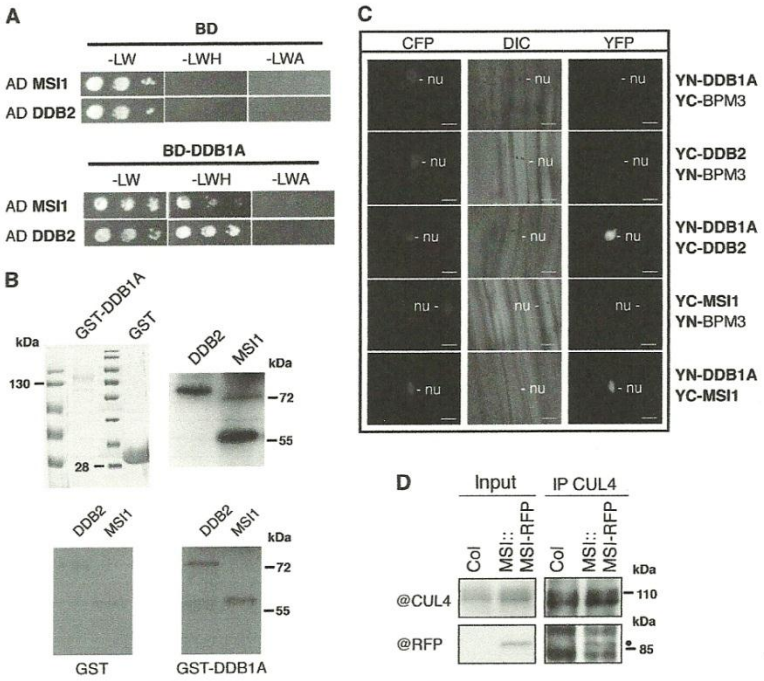


Figure 2 MSI1 forms a complex with DDB1A and CUL4. (**A**) Yeast two-hybrid experiments showing MSI1 interaction with DDB1A. Dilution series of yeast cells co-expressing the indicated proteins were grown for 3 days at 28°C on LWH (low-stringency selection) and on LWA (high-stringency selection). As a positive control, we used DDB2. (**B**) The interactions from the Y2H assay were confirmed by using bacterially expressed GST or GST-DDB1A proteins in pull down *in vitro* assays. Upper panel shows GST-DDB1A protein (left) and 5 µl of *in vitro* translated ^{35}S-Met-labelled MSI1 and DDB2 proteins (right) used for pull downs (lower panels). (**C**) BiFC of YN-DDB1A/YC-MSI1. Different combinations of plasmids expressing the indicated YN- and YC-fusion proteins were bombarded into hypocotyls of dark-grown mustard seedling. The nuclear-localized CUL3 receptor BPM3 protein was used here as a negative control. A transfection control CPRF2 expressing a fused CFP targeted to the nucleus (nu) was systematically included to identify transformed cells. Images were recorded 5 h after bombardment via CFP- (left panels) and YFP-specific filters (right panels). Differential interference contrast (DIC) images are shown (middle panels). Reconstitution of functional YFP as detected by YFP fluorescence occurs only in the nucleus with both MSI1 and DDB2. Scale bars = 20 µm. (**D**) *In vivo* pull down with CUL4 and MSI1. MSI1–RFP expressing and control wild-type plants were used for immunoprecipitation (IP) assays using anti-CUL4 antibody. Both CUL4 (upper right panel) and MSI1–RFP (lower right panel) were detected in the IPs, using anti-CUL4 and anti-RFP antibodies, respectively. An asterisk indicates the MSI1–RFP protein band. A full-colour version of this figure is available at *The EMBO Journal* Online.

ddb1b-1/ddb1b-1 (referred as *DDB1A/ddb1a ddb1b*) and *ddb1a-2/ddb1a-2 DDB1B/ddb1b-1* (referred as *ddb1a DDB1B/ddb1b*). Because both *ddb1a-2* and *ddb1b-1* mutants carry the same selection marker, we used PCR-based genotyping for further genetic analyses. Among the progeny of self-pollinated *DDB1A/ddb1a ddb1b* and *ddb1a DDB1B/ddb1b* plants, no double mutant were identified, despite the analysis of ~60 plants for each genotype (Table III).

Next, we evaluated the effect of both *DDB1*-related genes on male and female gametophytic transmission (Table III). Reciprocal crosses between the different genotypes revealed that the two genes do not contribute equally to gametophyte development and/or function as indicated by unequal transmission defects: while in the absence of *DDB1B*, *DDB1A* is required for normal transmission through the male, the converse is true for the female gametophyte.

The number of aborted seeds was consistent with zygotic embryo lethality in self-pollinated *ddb1a DDB1B/ddb1b* plants (Table II). Light microscopic observations of cleared seeds revealed that double homozygous *ddb1a dbb1b* embryos derived from selfed *DDB1A/ddb1a ddb1b* (not shown) or *ddb1a DDB1B/ddb1b* (Supplementary Figure S3D)) mutants arrest at the globular stage, with a phenotype reminiscent of that of the *cul4* mutants. Thus, both *CUL4* and *DDB1A/B* functions are required for normal development of embryo and endosperm.

CUL4 is expressed during embryogenesis

To determine the expression pattern of *CUL4* in reproductive tissues and during embryogenesis, we performed mRNA *in situ* hybridization experiments on sections of flower buds and developing siliques using *CUL4*-specific antisense and sense control probes. *CUL4* transcripts were detected in the tissues of young flower buds, that is in petals, stamens, and carpels (Supplementary Figure S4A). A distinctive signal was observed in emerging ovules (Supplementary Figure S4B), but not in the developing embryo sac (Supplementary Figure S4E). After fertilization, the expression level of *CUL4* was prominent in the developing embryo (Supplementary Figure S4C, D, G–L). The signal intensity decreased after the

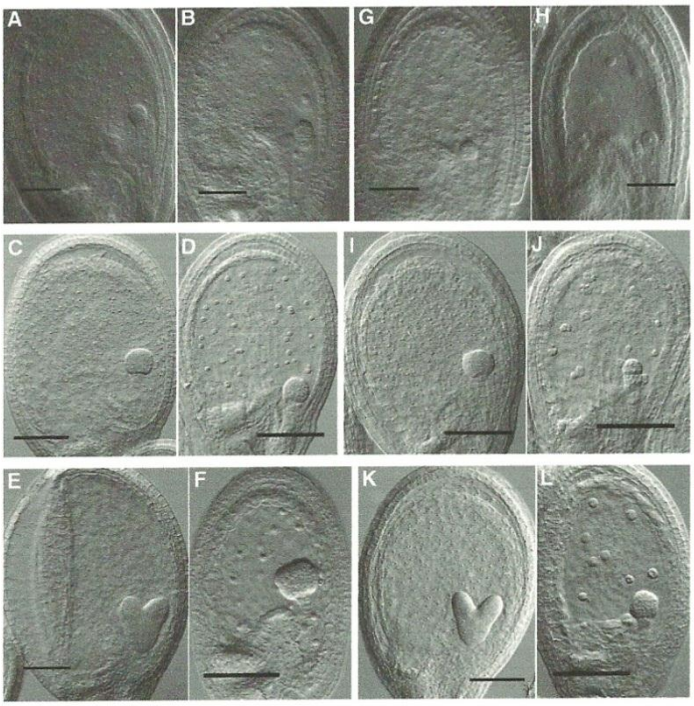


Figure 3 Embryo and endosperm development is affected in *cul4* mutant seeds. (**A**) Cleared seed with an embryo at the octant stage from the same *cul4-1* homozygous mutant silique as the seed shown in (**B**). (**B**) Mutant embryo and endosperm with reduced proliferation and large nuclei. (**C**) Cleared seed with an embryo at the globular stage from the same *cul4-1* homozygous mutant silique as the seed shown in (**D**). (**D**) Delayed mutant with large endosperm nuclei. (**E**) Cleared seed with an embryo at the heart stage from the same *cul4-1* homozygous mutant silique as the seed shown in (**F**). (**F**) Delayed mutant with reduced proliferation and enlarged endosperm nuclei. (**G**) Cleared seed with an embryo at the octant stage from the same silique as the seed shown in (**H**). (**H**) *cul4-2* homozygous mutant with a reduced number of large endosperm nuclei. (**I**) Cleared seed with an embryo at the globular stage from the same silique as the seed shown in (**J**). (**J**) Delayed *cul4-2* homozygous mutant with enlarged and aggregated endosperm nuclei. (**K**) Cleared seed with an embryo at the heart stage from the same silique as the seed shown in (**L**). (**L**) Delayed *cul4-2* homozygous mutant with a reduced number of enlarged endosperm nuclei. Bars = 50 μm (**A–B**, **G–H**); 100 μm (**C–F**, **I–L**).

Table I Genetic analysis of *cul4* mutant plants

Parental genotype (female × male)	Hyg^R	Hyg^s	*n*	*P*-value	TE_F (%)	TE_M (%)
cul4-2 (selfed)	470	296	766	0.002	NA	NA
cul4-3 (selfed)	474	236	710	0.936	NA	NA
Col-0 × cul4-2	213	251	464	0.077	NA	84.9%
cul4-2 × Col-0	198	236	434	0.068	83.9%	NA
Col-0 × cul4-3	229	256	485	0.220	NA	89.4%
cul4-3 × Col-0	221	254	475	0.130	87.0%	NA

Resistance to Hygromycin (Hyg^R, Hygromycin resistant seedlings; Hyg^S, Hygromycin sensitive seedlings) was used as a marker for the *cul4-2* and *cul4-3* insertions. Transmission efficiencies were calculated according to Howden *et al.* (1998): TE = Hyg^R/Hyg^S × 100%. *P*-value, based on a 2:1 segregation ratio as expected for a zygotic embryo lethal mutation and 1:1 for the reciprocal crosses as expected for normal transmission; TE_F, female transmission efficiency; TE_M, male transmission efficiency; NA, not applicable. At a *P*-value of <0.05 the null hypothesis is rejected.

heart stage (Supplementary Figure S4I–G). This expression pattern correlates with the requirement of *CUL4* for embryo development. In endosperm cells, the hybridization signal was low but detectable at all stages of seed development (Supplementary Figure S4C, D, G–L). Overall, the *CUL4* expression pattern in developing seeds is consistent with the *CUL4* loss-of-function phenotype.

Imprinted expression of MEA is lost in cul4 mutants

MSI1 is a component of the FIS–PRC2 complex, which is required for seed development (Köhler *et al*, 2003). However, MSI1 was also found in other protein complexes potentially involved in chromatin functions (Hennig *et al*, 2005). Thus, we wondered whether loss of *CUL4* and/or *DDB1* activity affects PRC2-like functions during plant reproduction.

Table II Analysis of mature siliques

Parental genotype (female × male)	Normal seeds	Aborted seeds	Seeds scored	*P*-value
Col-0 × *Col-0*	512	8 (1.5%)	520	NA
cul4-2$^{+/-}$ *(selfed)*	1129	444 (28.2%)	1573	0.003
cul4-3$^{+/-}$ *(selfed)*	1030	383 (27.1%)	1413	0.065
ddb1a-2 DDB1B/ddb1b-1 *(selfed)*	215	81 (27.4%)	296	0.347

Mature siliques were analysed for the presence of aborted seeds. *P*-value, based on a 3:1 ratio as expected for zygotic embryo lethality. NA, not applicable. At a *P*-value of <0.05 the null hypothesis is rejected.

Table III Genetic analysis of *ddb1a ddb1b* mutant plants

Genotyping	Doubly homozygous (−/− ; −/−)	Heterozygous for one allele (+/− ; −/−)	WT allele (+/+ ; −/−)	*n*	*P*-value	TE_F (%)	TE_M (%)
DDB1A/ddb1a-2 ddb1b-1 (selfed)	0	36	20	56	0.567	NA	NA
ddb1a-2 DDB1B/ddb1b-1 (selfed)	0	38	25	63	0.285	NA	NA
ddb1b-1 × DDB1A/ddb1a-2 ddb1b-1	0	26	68	94	<0.0001	NA	38.2%
DDB1A/ddb1a-2 ddb1b-1 × ddb1b-1	0	44	45	89	0.9156	97.8%	NA
ddb1a-2 × ddb1a-2 DDB1B/ddb1b-1	0	43	41	84	0.8273	NA	104.8%
ddb1a-2 DDB1B/ddb1b-1 × ddb1a-2	0	20	66	86	<0.0001	30.3%	NA

TE_F, female transmission efficiency; TE_M, male transmission efficiency; NA, not applicable.
The progeny of *DDB1A/ddb1a ddb1b* and *ddb1a DDB1B/ddb1b* plants were genotyped. No double mutant was identified. *P*-value, based on a 2:1 segregation ratio as expected for a zygotic embryo lethal mutation and 1:1 for the reciprocal crosses as expected for normal transmission. At a *P*-value of <0.05 the null hypothesis is rejected.

In contrast to the *msi1* mutant, *cul4-1* siliques did not show a clear elongation after emasculation (Supplementary Figure S5A). Nevertheless, we observed 16% ($n=229$) and 3.6% ($n=224$) of autonomous endosperm division in homozygote *cul4-1* and heterozygote *cul4-2* mutants, respectively (Supplementary Figure S5B). As expected, no extra divisions were observed in wild-type ovules ($n=71$) (Supplementary Figure S5B). Thus, although at a lower penetrance, *cul4* mutants share the *fis* class phenotype of endosperm initiation in the absence of fertilization (Ohad *et al*, 1996; Chaudhury *et al*, 1997; Grossniklaus and Vielle-Calzada, 1998; Kiyosue *et al*, 1999; Köhler *et al*, 2003; Guitton *et al*, 2004). Next, we investigated whether mutations of *CUL4* affect parental imprinting of *MEA* and/or *PHE1*, two genes that are regulated by the FIS–PRC2 complex (Köhler *et al*, 2003, 2005; Baroux *et al*, 2006; Gehring *et al*, 2006; Jullien *et al*, 2006). We used sequence polymorphisms between different Arabidopsis accessions to distinguish parental alleles. Interestingly, we detected paternal *MEA* expression in the *cul4-1* mutant (Figure 4A). It is noteworthy that repression of the paternal *MEA* allele in the control experiment was not complete, as a weak but detectable expression was observed when Columbia (Col-0) pollen was used. Indeed, previous genetic analyses suggested that Col-0 carries a paternal modifier of *mea* seed abortion (Vielle-Calzada *et al*, 1999), and it is possible that this leads to a weak de-repression of the paternal *MEA* allele. However, full expression of paternal *MEA* allele was only observed 3 days after pollination (DAP) when *cul4-1* or *cul4-1 ddb1a* pollen was used.

To further investigate the loss of repression of the paternal *MEA* allele in the *cul4-1* or *cul4-1 ddb1a* mutants, we introgressed the *pMEA::MEA-YFP* reporter gene (Wang *et al*, 2006a) into the *cul4-1* and *cul4-2* mutant backgrounds. When *pMEA::MEA-YFP* line in a Col-0 background was used to pollinate Col-0 plants, we observed by confocal microscopy a detectable fluorescence signal in the endosperm of about half of the seeds (Figure 4B; Table IV). This result is inconsistent with a full repression of the paternal *MEA* allele in the Col-0 background, but is in agreement with our results using sequence polymorphisms (see above). A similar result was observed when the *cul4-1* hypomorphic mutant was used as a female and pollinated with wild-type pollen carrying the *pMEA::MEA-YFP* reporter. However, when we used the *cul4-2 pMEA::MEA-YFP* pollen to fertilize Col-0 plants, we observed a stronger fluorescence signal in some seeds (Figure 4B), supporting a reactivation of the paternal *pMEA::MEA-YFP* reporter gene if derived from *cul4* mutant pollen.

To better deplete *CUL4* activity in such experiments, we combined the weak *cul4-1* with the strong *cul4-2* alleles. When homozygous *cul4-1* plants were used as a female and pollinated with pollen from *cul4-2* heterozygote plants, we observed a category of seeds (~50%), which arrested at the late globular stage, thus corresponding to *cul4-1/cul4-2* homozygous embryos. In these seeds, the underdeveloped endosperm contained large coenocytic cells (Supplementary Figure S6) and degenerated at 4 DAP. Interestingly, ~70% of seeds were scored for a fluorescent signal when the *cul4-1* mutant was pollinated with pollen from *cul4-2 pMEA::MEA-YFP* plants (Table IV). The fluorescence signal was particularly strong in the aberrant endosperm of arrested *cul4-1/cul4-2* seeds at 2 and 3 DAP (Figure 4B). This finding was further supported by the accumulation of paternally expressed MEA-YFP protein in this cross, as detected by western blotting (Figure 4C). Thus, our results indicate that *CUL4* is necessary to maintain repression of the paternal *MEA* allele.

Since the PRC2 complex mediates trimethylation of histone H3 at the lysine residue 27 (H3K27me3) (Gehring *et al*, 2006; Jullien *et al*, 2006; Makarevich *et al*, 2006), we investigated whether *CUL4* knockdown affects histone methylation. Interestingly, chromatin immunoprecipitation (ChIP) analysis performed on young siliques revealed a decrease in H3K27 trimethylation at the *MEA* locus (Figure 5B). It is remarkable that a similar effect was not observed when ChIP assays were performed with young floral buds (Figure 5A), suggesting that CUL4 is involved in the maintenance rather than in the establishment of those histone repressive marks.

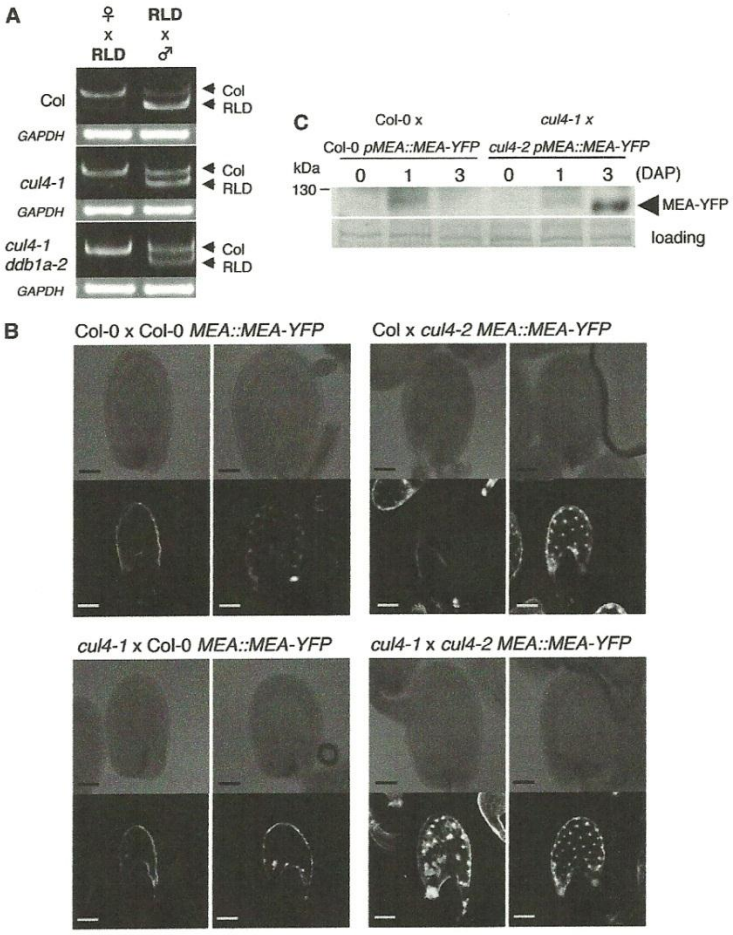


Figure 4 *MEA* parental imprinting is lost in *cul4* mutants. (**A**) Parental allele-specific expression analysis of *MEA* in wild-type, *cul4-1* and *cul4-1 ddb1a-2* seeds at 3 DAP. For *MEA* expression, Col-0 was crossed with the RLD accession. (**B**) Representative confocal and corresponding DIC images showing *pMEA::MEA-YFP* detection in various crosses at 2 DAP. A strong fluorescence signal was detected in the abnormally large coenocytic endosperm cells resulting from the *cul4-1 cul4-2 pMEA::MEA-YFP* crosses. Bar = 50 μm. (**C**) Paternally expressed MEA-YFP protein accumulation in two different crosses, as indicated. Protein extracts were analysed by immunoblotting using the anti-GFP antibody. Coomassie blue staining used as a loading control. A full-colour version of this figure is available at *The EMBO Journal* Online.

Table IV Paternal MEA-YFP signal detection in wild-type and *cul4* mutants

Parental genotype (female × male)	Negative	Positive (%)
Col-0 × Col-0 *MEA ::MEA-YFP*	14	15 (51.7%)
Col-0 × *cul4-2 MEA ::MEA-YFP*	13	15 (54.6%)
cul4-1 × Col-0 *MEA ::MEA-YFP*	13	14 (51.9%)
cul4-1 × *cul4-2 MEA ::MEA-YFP*	11	26 (70.2%)

In vegetative tissues, both alleles of *MEA* are silenced by PRC2 complexes through the deposition of H3K27me3 marks on chromatin (Gehring *et al*, 2006; Jullien *et al*, 2006). Thus, we tested whether CUL4 is also required to repress *MEA* later during development. Indeed, *MEA* expression was detected in homozygous *cul4-1* knockdown plants, though to a lesser extend than in a mutant compromised in the SET-domain protein CURLY LEAF (CLF) used here as control (Supplementary Figure S7). Moreover, ChIP analysis revealed that *MEA* reactivation was correlated with a decrease in H3K27 trimethylation (Supplementary Figure S7C).

PHE1 3′ region maintains maternal PHE1 repression in cul4 mutants

An intriguing observation was that in contrast to *MEA*, we did not observe the activation of the maternal *PHE1* allele in the *cul4* knockdown mutant when using the different Arabidopsis accessions (Figure 6A). Nevertheless, when examining H3K27 trimethylation at the *PHE1* locus (Figure 5), we observed a clear reduction in the repressive

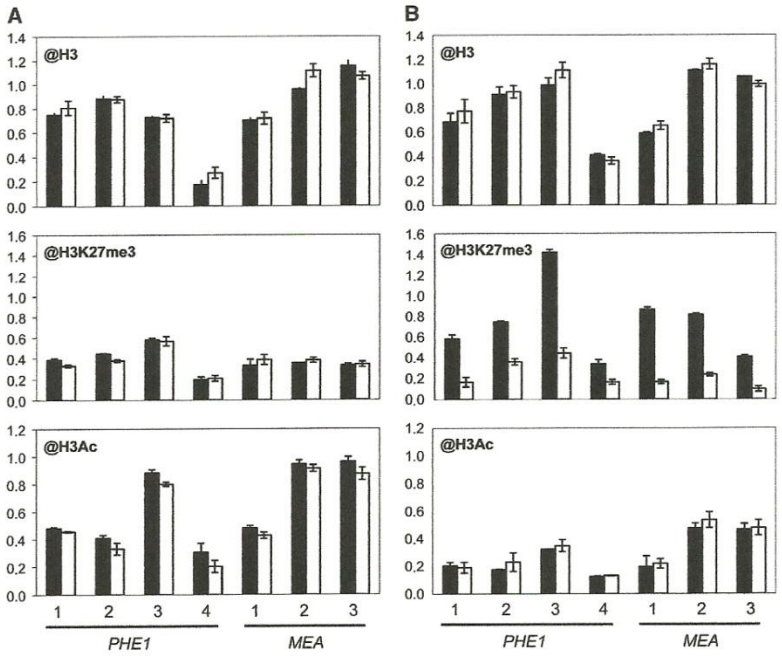


Figure 5 *CUL4* knockdown induces loss of the repressive H3K27me3 mark on both *MEA* and *PHE1* loci in young siliques. Relative levels of histone modifications on *PHE1* and *MEA* chromatin examined after ChIP assays using anti-H3K27me3 and anti-H3ac antibodies. Chromatin of Col-0 (black bars) and homozygous *cul4-1* mutant (white bars) was prepared from either closed floral buds prior to fertilization (**A**), or young siliques at 3–4 DAP (**B**). DNA fragments after ChIP were quantified by real-time quantitative PCR and were subsequently normalized to internal controls. Data shown are means ± s.d. of three technical replicates. Similar results were obtained in three independent experiments.

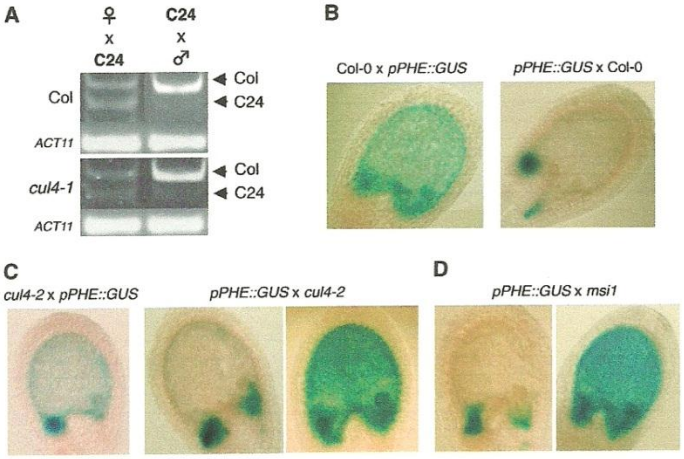


Figure 6 Paternally expressed CUL4 and MSI1 are required to maintain maternal repression of the *PHE1-GUS* reporter. (**A**) Parental allele-specific expression analysis of *PHE1* in wild-type and *cul4-1* seeds at 3 DAP. For *PHE1* expression, Col-0 was crossed with C24. (**B**) Expression of paternal (left panel) and maternal (right panel) *PHE1-GUS* transgene in wild-type seeds 3 DAP. (**C**) Expression of maternal (middle and right panels) *PHE1-GUS* transgene 3 DAP using *cul4-2* mutant pollen. Seeds presenting a strong GUS staining (~50%) correspond to *cul4-2* heterozygote genotype. (**D**) Expression of maternal *PHE1-GUS* transgene 3 DAP using *msi1* mutant pollen. Seeds presenting a strong GUS staining (~50%) correspond to *msi1* heterozygotes.

histone marks, to a similar level as at the *MEA* locus. Although parental imprinting of both genes requires the FIS–PRC2, it was however recently found that *Polycomb*-dependent silencing is necessary but not sufficient to establish *PHE1* imprinting. Indeed, a distantly located region downstream of the *PHE1* locus, named DMR (differentially

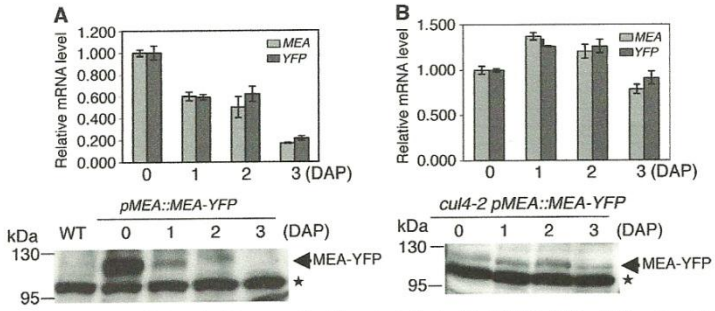


Figure 7 MEA expression in developing fruits of wild-type and *cul4* mutant plants. (**A**, **B**) MEA-YFP mRNA and protein levels in developing fruits of self-fertilized Col-0 (**A**) and *cul4-2* heterozygote mutant plants (**B**). Relative levels of total *MEA* (blue bars) and *MEA-YFP* (red bars) transcripts determined by quantitative RT–PCR are shown on the upper panels. The transcript level of MEA-YFP was reduced by ~50% in *cul4-2* when compared with Col-0 at 0 DAP (not shown). Data are means ± s.d. MEA-YFP protein levels are shown in the lower part. A measure of 40 μg of total protein extracts was analysed by immunoblotting using an anti-GFP antibody. A full-colour version of this figure is available at *The EMBO Journal* Online.

methylated region), has also an important function in silencing the maternal *PHE1* allele (Makarevich *et al*, 2008). Thus, it is possible that the reduction in repressive histone methylation marks seen in *cul4-1* is not sufficient to impair maternal *PHE1* silencing. To examine this possibility, we took advantage of the *PHE1-GUS* reporter construct, which contains the ~3 kbp promoter sequence but no 3′ regulatory elements (Köhler *et al*, 2003). The paternally derived PHE1-GUS transgene was similarly expressed in wild-type ($n=55$; Figure 6B) and *cul4-2* heterozygote mutant seeds ($n=49$; Figure 6C). Expression of the maternally derived *PHE1-GUS* was mainly restricted to the chalazal endosperm when pollinated with wild-type pollen (Figure 6B). In contrast, 3 DAP with heterozygote *cul4-2* pollen, the maternally derived *PHE-GUS* transgene was expressed at a higher level in about half of the seeds (56 out of 118 seeds exhibited GUS staining in the endosperm; Figure 6C). A similar result was observed with pollen from heterozygote *msi1* mutant plants (78 out of 148 seeds exhibited GUS staining in the endosperm; Figure 6D). Thus, paternally expressed CUL4 and MSI1 are both required to maintain the silencing of the maternal *PHE1-GUS* reporter, suggesting haplo-insufficiency. It is, therefore, likely that the silencing of endogenous maternal *PHE1* is maintained in *cul4* mutant seeds due to additional regulatory elements, most likely located in the 3′ region of the gene (Makarevich *et al*, 2008).

MEA transcript and protein accumulate in CUL4-deficient seeds

Next, we investigated the kinetics of both *MEA* transcripts and protein accumulation. The Col-0 *pMEA::MEA-YFP* line was self-pollinated and MEA-YFP mRNA and protein levels were determined before and during the first 3 DAP. While the *MEA* mRNA level gradually decreased after pollination, MEA protein quickly disappeared and was hardly detectable at 2 DAP (Figure 7A). Thus, the MEA protein level is highly dynamic and seems under both transcriptional and post-transcriptional control. Therefore, we investigated MEA-YFP protein accumulation in *cul4* mutant plants. It is noteworthy that the *pMEA::MEA-YFP* transgene was partially silenced in the *cul4-2* background. This phenomenon, at least in part, could explain the lower MEA-YFP protein content detected in the mutant (Figure 7B). Since *MEA* transcript levels did not decay in the *cul4-2* mutant (Figure 7B), it seems that the MEA-YFP protein accumulation mainly reflects transcriptional regulation by CUL4, though a post-transcriptional regulation cannot be excluded. Nevertheless, our results clearly demonstrate that *CUL4* activity is necessary to restrict *MEA* expression during seed development.

Discussion

MSI1 is a well-characterized WD40 protein with several functions in the control of chromatin dynamics and gene expression (Hennig *et al*, 2005). In particular, MSI1 was identified as a subunit of the FIS–PRC2 complex (Köhler *et al*, 2003), which regulates parental imprinting during seed development. Here, we provide evidence that CUL4–DDB1 physically associates with MSI1 and is involved in the regulation of the FIS–PRC2 in Arabidopsis. However, because the MSI1 protein associates with additional protein complexes such as chromatin assembling factor 1 (CAF-1) and a complex with the retinoblastoma-related protein (Exner *et al*, 2006; Jullien *et al*, 2008), we do not exclude the possibility that CUL4–DDB1 acts at more than one level.

In flowering plants, imprinting has been mostly studied in the endosperm, which is a terminal tissue developing after fertilization of the central cell (reviewed in Grossniklaus, 2005; Feil and Berger, 2007; Köhler and Weinhofer-Molisch, 2010). Thus far, several genes have been found to be maternally expressed but paternally silenced including *MEA*, *FIS2* and *FWA* (Vielle-Calzada *et al*, 1999; Kinoshita *et al*, 1999, 2004; Jullien *et al*, 2006). In particular, the paternal silencing of *MEA* requires the activity of the FIS–PRC2, which mediates trimethylation of histone H3 at the lysine residue 27 (H3K27me3) (Gehring *et al*, 2006; Jullien *et al*, 2006; Makarevich *et al*, 2006). In contrast, the MADS-box gene *PHE1* is predominantly expressed from the paternal allele while the maternal allele is downregulated (Köhler *et al*, 2005). The maternal *PHE1* allele is repressed through the combined action of the FIS–PRC2 containing MEA and the unmethylated DNA state of a DMR in the 3′ region of *PHE1* (Köhler *et al*, 2005; Makarevich *et al*, 2006). In sperm cells, the DMR is most likely methylated by the maintenance DNA

methyltransferase MET1, preventing silencing and leading to an active paternal *PHE1* allele (Makarevich *et al*, 2008).

In line with a role of CUL4–DDB1 in the regulation of the FIS–PRC2 complex, we could show that reduced *CUL4* activity leads to autonomous endosperm division, although at a lower penetrance than in some other *fis* class mutants (Ohad *et al*, 1996; Chaudhury *et al*, 1997; Köhler *et al*, 2003; Guitton *et al*, 2004). However, the percentage of seeds with a *fis* phenotype observed in *cul4* (3.6–16%) is rather similar to that of *mea* mutants ranging from 3 to 20% (Grossniklaus and Vielle-Calzada, 1998; Kiyosue *et al*, 1999). For *mea* this low penetrance can be explained due to functional redundancy with its paralogue *SWINGER* (Wang *et al*, 2006a). Moreover, we showed that paternal *MEA* silencing was lost when *CUL4* function was compromised. Consistently, H3K27me3 repressive marks were significantly reduced in the *cul4-1* knockdown mutant. A similar reduction in H3K27me3 marks was also observed at the *PHE1* locus, albeit this was not sufficient to abolish downregulation of the maternal *PHE1* allele, most likely because of the presence of additional regulatory mechanisms depending on DNA methylation (Makarevich *et al*, 2008).

Several observations suggest that CUL4–DDB1 is not required for the establishment of *MEA* paternal silencing, but rather for its maintenance. First, we never observed paternal *MEA* reactivation in the pollen of *cul4* knockdown or null mutants (data not shown). Second, we only visualized a strong paternal *MEA* expression 2–3 DAP, but not at 1 DAP. Third, ChIP experiments failed to reveal a loss of H3K27me3 marks at the *MEA* locus in the young floral buds, but only became evident after fertilization in young siliques. Finally, we also found that *CUL4* participates in the maintenance of *MEA* repression at a later developmental stage (e.g. in 17-day-old plants), which depends on another form of PRC2 containing CLF as the histone methyltransferase.

Whether CUL4 and DDB1 only associate transiently or are more stable components of the FIS–PRC2 complex will need further investigations. However, it is noteworthy that MSI1 together with FIE, MEA and FIS2 were found in a very large protein complex of about 650 kDa (Köhler *et al*, 2003), leading the authors to speculate that other proteins associate with FIS–PRC2. Another intriguing observation is that the PRC2 core component FIE, which is also part of various other forms of PRC2 in Arabidopsis (Pien and Grossniklaus, 2007), was predicted to interact with DDB1 based on its structure (Lee *et al*, 2008). Thus, not only one but even two PRC2 components may recruit CUL4 to the FIS–PRC2. Moreover, because metazoan homologs of Arabidopsis MSI1-like proteins, such as the retinoblastoma-binding proteins P55 in Drosophila and RbAp48 in mammals, have structural features of typical CUL4 substrate receptors, it is probable that our findings will extend to other organisms beyond plants.

Although CUL4–DDB1 is involved in FIS–PRC2 functions, *CUL4* loss-of-function mutations do not phenocopy all aspects of *fis* class mutants. In particular, we did not observe in *cul4* mutants as strong penetrance of autonomous endosperm development as in *msi1* mutants (Köhler *et al*, 2003; Guitton *et al*, 2004), nor the presence of parthenogenetic embryos (Guitton and Berger, 2005). This could be explained by at least two different features that distinguish *cul4* from the *fis* class mutants. First, recent work suggests that Arabidopsis *CUL4* is also involved in cell-cycle regulation (Marrocco *et al*, 2010; Roodbarkelari *et al*, 2010), as it has been shown in metazoans (Jin *et al*, 2006; Abbas *et al*, 2008; Havens and Walter, 2009). Thus, instead to promote cell proliferation in the endosperm as observed in the *fis* class mutants, the loss of *CUL4* restricts cell division in this tissue counteracting its *fis* phenotype. Second, the *cul4-1* knockdown does not affect parental imprinting of all FIS–PRC2 targets. In particular, we did not observe the de-repression of the maternal *PHE1* allele, although we found a decrease in H3K27 methylation at this locus. *PHE1* encodes a MADS-domain transcription factor (Köhler *et al*, 2003), while misexpression in *fis* class mutants, but not in *cul4-1*, could explain some phenotypic differences during seed development.

Ubiquitylation has already been linked to *Polycomb*-mediated repression (reviewed in Niessen *et al*, 2009). Indeed, the human PRC1 complex exhibits an E3 ligase activity for histone H2A (Wang *et al*, 2004), which is triggered by two of its subunits, RING1 and RNF2 (also referred to RING1B or RING2) (de Napoles *et al*, 2004; Buchwald *et al*, 2006). In the prevailing model, PRC1 binds to histone H3K27me3 to catalyse monoubiquitination of histone H2A, which in turn could interfere with the transcriptional machinery or chromatin remodelling proteins to repress transcription of target genes (Stock *et al*, 2007; Zhou *et al*, 2008). Moreover, it was recently shown that a tight balance between histone H2A ubiquitylation and deubiquitylation is important for *Polycomb*-mediated repression in Drosophila (Scheuermann *et al*, 2010). Arabidopsis also contains RING-domain proteins that, together with LHP1, may fulfil PRC1-like functions (Xu and Shen, 2008), most likely via histone H2A ubiquitylation (Bratzel *et al*, 2010).

Our finding that the CUL4–DDB1^{MSI1} E3 ligase is required in the maintenance of FIS–PRC2-dependent parental imprinting in Arabidopsis raises the question which substrate(s) are targeted for ubiquitylation in this process. One possibility is that CUL4–DDB1^{MSI1} ubiquitylates directly one of the FIS–PRC2 subunits.

We initially speculated that MSI1 could be either a substrate or a substrate receptor of this E3 ligase, or even both. Thus, we introgressed the *pMSI::MSI1–RFP* reporter gene (Chen *et al*, 2008) into the *cul4-1* mutant background and checked for MSI1 protein accumulation. However, when *cul4-1 pMSI::MSI1–RFP* plants were self-pollinated, the MSI1–RFP protein level was only slightly higher than in a wild-type background (Supplementary Figure S8), suggesting that MSI1 protein turnover is not controlled by CUL4.

In contrast, we noticed that MEA protein does not decay after pollination in *cul4* mutants. Thus, it is possible that ubiquitylation controls its stability, although we cannot exclude that this accumulation mainly results from the persistence of the *MEA* transcript. Nevertheless, unscheduled MEA protein accumulation may alter FIS–PRC2 activity by, for example, titering some of its components or associated proteins. This may also explain the paradox why in the presence of more MEA protein, repression of the paternal *MEA* allele is lost.

Finally, it is also possible that CUL4–DDB1^{MSI1} acts at the FIS–PRC2 level by a mechanism that does not imply protein degradation. In this respect, it is well established that CRL4 E3 ligases trigger different kinds of non-proteolytic ubiquitylation reactions, including the assembly of K63-linked polyubiquitin chains and monoubiquitylation. Thus,

in the process of nucleotide excision repair after UV damage, a CUL4–DDB1^{DDB2} E3 ligase (DDB2 being a WD40 substrate receptor) triggers non-proteolytic ubiquitylation of XPC (xeroderma pigmentosum complementation group C) to permit its binding to damaged DNA (Sugasawa *et al*, 2005) where it induces histone ubiquitylation, presumably to modify the chromatin structure at the sites of DNA lesions (Kapetanaki *et al*, 2006; Wang *et al*, 2006b). Interestingly, the fission yeast Cul4 associates with Clr4 histone methyltransferase and is required for RNAi-mediated heterochromatin formation (Hong *et al*, 2005; Jia *et al*, 2005), although the CUL4-mediated modifications, which are involved in this process, remain unknown. Future investigations will identify CUL4–DDB1^{MSI1} E3 substrates and clarify its function(s) in PRC2-dependent epigenetic regulation.

Materials and methods

Plant material and growth conditions

The Arabidopsis *ddb1a-2* mutant is described in Molinier *et al* (2008). The *ddb1b-1* (SALK 061944) mutant was identified using the web-assisted program at http://signal.salk.edu/cgi-bin/tdnaexpress. The precise location of the T-DNA in *ddb1b-1* mutant was determined by sequencing, showing an insertion after nucleotide 6211 in the last exon of *DDB1B*. The *cul4-1* (GABI-KAT 600H03) mutant is described in Bernhardt *et al* (2006). The *cul4-2* (Koncz 42460) and *cul4-3* (Koncz 57891) mutants were identified by PCR screening of the Köln Arabidopsis T-DNA mutant collection (Ríos *et al*, 2002). The precise location of the T-DNA in *cul4-2* and *cul4-3* was determined by sequencing, showing an insertion after nucleotide 4478 in the 14th exon and after nucleotide 2718 in the 7th intron of *CUL4*, respectively.

For *in vitro* culture, seeds were surface sterilized using the ethanol method, plated on GM medium (MS salts (Duchefa, The Netherlands), 1% sucrose, 0.8% agar, pH 5.8) in the presence or absence of a selectable agent, stored 2 to 3 days at 4°C in the dark, and then transferred to a plant growth chamber under a 16-h/8-h photoperiod (22uC/20uC). For soil-cultured plants, seeds were sown (20/pot) and put at 4°C in the dark during 3 days. Two weeks later, single plants were transferred to pots in the greenhouse and kept under a regime of 16 h/8 h photoperiod (20uC/16uC; 70% humidity).

Yeast two-hybrid assays

The DDB2 and MSI1 cDNAs were cloned as fusions to the GAL4 activation domain and the DBB1A cDNA fused to the GAL4-binding domain, respectively, in Gateway-compatible pGAD424gate and pGBT9gate (Ghent plasmids collection, http://bccm.belspo.be/index.php) yeast two-hybrid vectors. The yeast strain AH109 (Clontech) was transformed with the appropriate combinations of bait and prey vectors. Transformants were selected on synthetic (SD)/-Leu/-Trp (-LW) media and interactions were tested on SD/-Leu/-Trp/-His (-LWH) or SD/-Leu/-Trp/-Ade (-LWA) media, allowing growth for 4 days at 28°C.

GST pull-down assay

The full-length DDB1A cDNA was cloned into Gateway vector pDEST15 (Invitrogen) by recombination for expression in *Escherichia coli* BL21AI (Invitrogen). In this construct GST is placed in frame at the N-terminus of DDB1A protein. After 4 h of 0.2% Arabinose induction at 16°C, the fusion proteins were purified on bulk gluthatione-sepharose following the manufacturer's instructions (GE Healthcare). For GST pull-down assays, DDB2 and MSI1 proteins were translated *in vitro*, using the TNT7-coupled wheat germ extract system (Promega) and radiolabelled with [^{35}S]-methionine. Purified GST-DDB1A or control GST proteins, immobilized on glutathione-sepharose beads, were incubated for 2 h at 4°C with equal amounts of ^{35}S-methionine-labelled DDB2 and MSI1 protein following the manufacturer's instructions (GE Healthcare). Labelled DDB2 and MSI1 proteins were detected by autoradiography.

Bimolecular fluorescence complementation

The DDB1A, DDB2 and MSI1 coding sequences were cloned into the split YFP destination vectors by recombination (Invitrogen) in order to obtain the YN-DDB1A, YC-DDB2 and YC-MSI1 constructs. The YN-, YC- and ^{35}S-CPRF2-CFP vectors and split YFP experiments were performed as described in Stolpe *et al* (2005). Vectors bearing YN or YC either alone or fused to BPM3 (At2g39760) were used as negative controls. Images were recorded 20 h after bombardment with a Nikon fluorescent stereomicroscope E800 equipped with a 40× water immersion optic by using CFP- and YFP-specific filters.

Immunoprecipitation experiments

Total soluble proteins were extracted from pMSI1::MSI1–RFP plants (Chen *et al*, 2008) using buffer A (100 mM $NaHPO_4$ pH 8.0, 1% Triton × 100, protease inhibitor mix (Complete; Roche Molecular Biochemical)). Immunoprecipitation assay was performed using anti-CUL4 polyclonal antibody coupled to ProteinA-sepharose beads. The MSI1–RFP protein was detected using anti-DsRed antibodies (Clontech Laboratories Cat#632496) diluted 1:1000 (v:v).

Histology and microscopy

Developing seeds were prepared from siliques of different developmental stages and directly mounted on microscope slides in a clearing solution of 8:2:1 chloral hydrate:distilled water:glycerol as described in Grini *et al* (2002). Observations were performed with a Zeiss Axiophot or a Leica DMR microscope under differential interference contrast (DIC) ×20 and ×40 optics. For YFP marker analysis, seeds from dissected siliques at different DAP were mounted in a 1:10 glycerol:distilled water. Specimens were observed under a Zeiss confocal laser-scanning microscope. Histochemical assays to detect GUS activity were performed as described in Capron *et al* (2003).

In situ hybridization

In situ hybridization was performed on the 10-μm Paraplast Plus (Sigma) sections as described in Brukhin *et al* (2005). For probe synthesis, the fragment spanning the region of the CUL4 (At5g46210) cDNA from 1501 to 2000 (500 bp) sequence was used. The fragment was inserted into the pGemT plasmid (Promega). Sense and antisense digoxygenin-UTP-labelled riboprobes were generated by run-off transcription using T7 and Sp6 RNA polymerases, respectively. The probes were hydrolyzed into 120 bp fragments in carbonate buffer, pH = 10.2 for 59 min at 60°C.

RNA extraction and RT–PCR analysis

RNAs from siliques at 3 DAP and from 17-day-old plantlets were prepared with the Trizol reagent (Invitrogen). In all, 5 μg of total RNA was treated with Dnase1 kit (Fermentas) and reverse transcribed with Superscript III Reverse Transcriptase kit (Invitrogen). To detect *PHE1* and *MEA* mRNA, allele-specific RT–PCR was performed as described previously in Kinoshita *et al* (1999) and Köhler *et al* (2005). Primers used to amplify the control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA, were GAPDH3′ (5′-GTAGCCCCACTCGTTGTCGTA-3′) and GAPDH5′ (5′-AGGGTGGT GCCAAGAAGGTTG-3′). To detect DDB1B, specific primers DDB1FWD (5′GGAAAATGAACCAACTAAGGAAGG-3′) and DDB1bREV (5′AGAG CTTGGATTTGCTTCAGTG-3′) were used. To detect EF1α-specific primers EF1Fwd (5′-TTGCTCCACAGGATTGACCACTG-3′) and EF1Rev 5′-TCACTTCGCACCCTTCTTGACG-3′) were used.

Quantitative PCR

Total RNA for quantitative PCR (qPCR) was extracted from inflorescences and siliques at different DAP of pMEA::MEA-YFP plants in Col-0 and *cul4-2* heterozygous mutant backgrounds, respectively, using the kit RNeasy MINI PLUS (Qiagen). In all, 2 μg of total RNA was reverse transcribed with Superscript III Reverse Transcriptase kit (Invitrogen). PCR was performed using gene-specific primers in a total volume of 10 μl SYBR Green Master mix (Roche) on a Lightcycler LC480 apparatus (Roche) according to the manufacturer's instructions.

The mean value of three replicates was normalized using the ACTIN2 (AT3G18780), GAPDH (AT3G26650) genes as internal controls.

Primer list:

MEA: GCAGGACTATGGTTTGGATG and CACCTTGAGGTAACA ATGCTC

YFP: ATATCATGGCCGACAAGCA and GAACTCCAGCAGGACCA TGT
ACTIN2: CTTGCACCAAGCAGCATGAA and CCGATCCAGACAC TGTACTTCCTT
GAPDH: TTGGTGACAACAGGTCAAGCA and AAACTTGTCGCTC AATGCAATC

Chromatin immunoprecipitation

ChIP experiments were performed as described by Jiang *et al* (2008) using young flowers before fertilization and young siliques around 3 DAP as well as 17-day-old plants. A measure of 5 ml mixed tissue powder was prepared after cross-linking DNA with proteins by formaldehyde. Preparation of chromatin, sonication, and immunoprecipitation using anti-H3 (05-499; Millipore), anti-trimethyl-histone H3K27 (07-449; Millipore) and anti-acetyl-H3 (06-599; Millipore) antibodies were carried out using Millipore ChIP kit according to the manufacturer's instructions. The immunoprecipitated DNA was analysed by real-time qPCR using *MEA* primers (region −700–1500; Baroux *et al*, 2006) and *PHE1* primers (Makarevich *et al*, 2006). *FUSCA* primers (Kwon *et al*, 2009) were used as internal standards for normalization. Data analysis was done as described in Mutskov and Felsenfeld (2004). Experiment was performed three times using independent biological samples.

Protein extraction and immunoblotting

Total proteins were extracted from Col-0 and *cul4* siliques at 1, 2 and 3 DAP using denaturating buffer as described in Büche *et al* (2000). A measure of 40 μg of total protein extracts were separated on SDS–PAGE gels and blotted onto Immobilon-P membrane (Millipore). The MSI1–RFP protein was detected by using the anti-DsRed antibody (Clontech Laboratories Cat#632496) diluted 1:1000 (v:v), whereas the MEA-YFP protein was identified by using a Rabbit anti-GFP polyclonal antibody diluted 1:10 000 (v/v).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We acknowledge M Bureau for help with confocal imaging, C Baroux for help in analysing *cul4* mutant seed defects, F Berger for providing several reporter constructs. Funding was provided by the CNRS, the French National Research Agency ANR (BLAN07-3_188961) and the EU NoE (Rubicon LSHG-CT-2005-018683). Work in UG's laboratory was supported by the University of Zürich, the Novartis Foundation and the Swiss National Science Foundation (31-112489).

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abbas T, Sivaprasad U, Terai K, Amador V, Pagano M, Dutta A (2008) PCNA-dependent regulation of p21 ubiquitylation and degradation via the CRL4Cdt2 ubiquitin ligase complex. *Genes Dev* **22:** 2496–2506

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H *et al* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301:** 653–657

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410

Angers S, Li T, Yi X, MacCoss MJ, Moon RT, Zheng N (2006) Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. *Nature* **443:** 590–593

Baroux C, Gagliardini V, Page DR, Grossniklaus U (2006) Dynamic regulatory interactions of *Polycomb* group genes: *MEDEA* autoregulation is required for imprinted gene expression in *Arabidopsis*. *Genes Dev* **20:** 1081–1086

Bernhardt A, Lechner E, Hano P, Schade V, Dieterle M, Anders M, Dubin MJ, Benvenuto G, Bowler C, Genschik P, Hellmann H (2006) CUL4 associates with DDB1 and DET1 and its downregulation affects diverse aspects of development in *Arabidopsis thaliana*. *Plant J* **47:** 591–603

Büche C, Poppe C, Schäfer E, Kretsch T (2000) *eid1*: a new *Arabidopsis* mutant hypersensitive in phytochrome A-dependent high-irradiance responses. *Plant Cell* **12:** 547–558

Buchwald G, van der Stoop P, Weichenrieder O, Perrakis A, van Lohuizen M, Sixma TK (2006) Structure and E3-ligase activity of the ring-ring complex of *Polycomb* proteins Bmi1 and Ring1b. *EMBO J* **25:** 2465–2474

Bratzel F, López-Torrejón G, Koch M, Del Pozo JC, Calonje M (2010) Keeping cell identity in *Arabidopsis* requires PRC1 RING-finger homologs that catalyze H2A monoubiquitination. *Curr Biol* **20:** 1853–1859

Brukhin V, Gheyselinck J, Gagliardini V, Genschik P, Grossniklaus U (2005) The RPN1 subunit of the 26S proteasome in *Arabidopsis* is essential for embryogenesis. *Plant Cell* **17:** 2723–2737

Capron A, Serralbo O, Fülöp K, Frugier F, Parmentier Y, Dong A, Lecureuil A, Guerche P, Kondorosi E, Scheres B, Genschik P (2003) The *Arabidopsis* anaphase-promoting complex or cyclosome: molecular and genetic characterization of the APC2 subunit. *Plant Cell* **15:** 2370–2382

Chaudhury AM, Ming L, Miller C, Craig S, Dennis ES, Peacock WJ (1997) Fertilization-independent seed development in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **94:** 4223–4228

Chen Z, Tan JL, Ingouff M, Sundaresan V, Berger F (2008) Chromatin Assembly Factor1 regulates the cell cycle but not cell fate during male gametogenesis in *Arabidopsis thaliana*. *Development* **135:** 65–73

Ciechanover A, Orian A, Schwartz AL (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* **22:** 442–451

de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, Nesterova TB, Silva J, Otte AP, Vidal M, Koseki H, Brockdorff N (2004) *Polycomb* group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. *Dev Cell* **7:** 663–676

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32:** 1792–1797

Exner V, Taranto P, Schönrock N, Gruissem W, Hennig L (2006) Chromatin assembly factor CAF-1 is required for cellular differentiation during plant development. *Development* **133:** 4163–4172

Feil R, Berger F (2007) Convergent evolution of genomic imprinting in plants and mammals. *Trends Genet* **23:** 192–199

Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, Harada JJ, Goldberg RB, Fischer RL (2006) DEMETER DNA glycosylase establishes *MEDEA Polycomb* gene self-imprinting by allele-specific demethylation. *Cell* **124:** 495–506

Grini PE, Jürgens G, Hülskamp M (2002) Embryo and endosperm development is disrupted in the female gametophytic *capulet* mutants of *Arabidopsis*. *Genetics* **162:** 1911–1925

Grossniklaus U (2005) Genomic imprinting in plants: a predominantly maternal affair. In *Annual Plant Reviews: Plant Epigenetics*, (Meyer P, ed), Blackwell, Sheffield, UK pp. 174–200

Grossniklaus U, Vielle-Calzada J-P (1998) Parental conflict and infanticide during embryogenesis. *Trends Plant Sci* **3:** 328

Grossniklaus U, Vielle-Calzada J-P, Hoeppner MA, Gagliano WB (1998) Maternal control of embryogenesis by *MEDEA*, a *Polycomb* group gene in *Arabidopsis*. *Science* **280:** 446–450

Guitton AE, Page DR, Chambrier P, Lionnet C, Faure JE, Grossniklaus U, Berger F (2004) Identification of new members of FERTILISATION INDEPENDENT SEED *Polycomb* group pathway involved in the control of seed development in *Arabidopsis thaliana*. *Development* **131:** 2971–2981

Guitton AE, Berger F (2005) Loss of function of *MULTICOPY SUPPRESSOR OF IRA1* produces nonviable parthenogenetic embryos in *Arabidopsis*. *Curr Biol* **15:** 750–754

Havens CG, Walter JC (2009) Docking of a specialized PIP Box onto chromatin-bound PCNA creates a degron for the ubiquitin ligase CRL4Cdt2. *Mol Cell* **35:** 93–104

He YJ, McCall CM, Hu J, Zeng Y, Xiong Y (2006) DDB1 functions as a linker to recruit receptor WD40 proteins to CUL4-ROC1 ubiquitin ligases. *Genes Dev* **20:** 2949–2954

Hennig L, Bouveret R, Gruissem W (2005) MSI1-like proteins: an escort service for chromatin assembly and remodeling complexes. *Trends Cell Biol* **15:** 295–302

Higa LA, Wu M, Ye T, Kobayashi R, Sun H, Zhang H (2006) CUL4-DDB1 ubiquitin ligase interacts with multiple WD40-repeat proteins and regulates histone methylation. *Nat Cell Biol* **8:** 1277–1283

Higa LA, Zhang H (2007) Stealing the spotlight: CUL4-DDB1 ubiquitin ligase docks WD40-repeat proteins to destroy. *Cell Div* **2:** 5

Hong EJ, Villén J, Gerace EL, Gygi SP, Moazed D (2005) A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. *RNA Biol* **2:** 106–111

Howden R, Park SK, Moore JM, Orme J, Grossniklaus U, Twell D (1998) Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in Arabidopsis. *Genetics* **149:** 621–631

Jia S, Kobayashi R, Grewal SI (2005) Ubiquitin ligase component Cul4 associates with Clr4 histone methyltransferase to assemble heterochromatin. *Nat Cell Biol* **7:** 1007–1013

Jiang D, Wang Y, Wang Y, He Y (2008) Repression of *FLOWERING LOCUS C* and *FLOWERING LOCUS T* by the *Arabidopsis Polycomb* repressive complex 2 components. *PLoS One* **3:** e3404

Jin J, Arias EE, Chen J, Harper JW, Walter JC (2006) A family of diverse Cul4-Ddb1-interacting proteins includes Cdt2, which is required for S phase destruction of the replication factor Cdt1. *Mol Cell* **23:** 709–721

Jullien PE, Katz A, Oliva M, Ohad N, Berger F (2006) *Polycomb* group complexes self-regulate imprinting of the *Polycomb* group gene *MEDEA* in *Arabidopsis*. *Curr Biol* **16:** 486–492

Jullien PE, Mosquna A, Ingouff M, Sakata T, Ohad N, Berger F (2008) Retinoblastoma and its binding partner MSI1 control imprinting in *Arabidopsis*. *PLoS Biol* **6:** e194

Kapetanaki MG, Guerrero-Santoro J, Bisi DC, Hsieh CL, Iæ-Otrin V, Levine AS (2006) The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in Xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. *Proc Natl Acad Sci USA* **103:** 2588–2593

Kinoshita T, Yadegari R, Harada JJ, Goldberg RB, Fischer RL (1999) Imprinting of the *MEDEA Polycomb* gene in the *Arabidopsis* endosperm. *Plant Cell* **11:** 1945–1952

Kinoshita T, Miura A, Choi Y, Kinoshita Y, Cao X, Jacobsen SE, Fischer RL, Kakutani T (2004) One-way control of *FWA* imprinting in *Arabidopsis* endosperm by DNA methylation. *Science* **303:** 521–523

Kiyosue T, Ohad N, Yadegari R, Hannon M, Dinneny J, Wells D, Katz A, Margossian L, Harada JJ, Goldberg RB, Fischer RL (1999) Control of fertilization-independent endosperm development by the *MEDEA Polycomb* gene in *Arabidopsis*. *Proc Natl Acad Sci USA* **96:** 4186–4191

Köhler C, Hennig L, Bouveret R, Gheyselinck J, Grossniklaus U, Gruissem W (2003) *Arabidopsis* MSI1 is a component of the MEA/FIE *Polycomb* group complex and required for seed development. *EMBO J* **22:** 4804–4814

Köhler C, Page DR, Gagliardini V, Grossniklaus U (2005) The *Arabidopsis thaliana* MEDEA *Polycomb* group protein controls expression of *PHERES1* by parental imprinting. *Nat Genet* **37:** 28–30

Köhler C, Weinhofer-Molisch I (2010) Mechanisms and evolution of genomic imprinting in plants. *Heredity* **105:** 57–63

Kwon CS, Lee D, Choi G, Chung WI (2009) Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in *Arabidopsis*. *Plant J* **60:** 112–121

Lee JH, Terzaghi W, Gusmaroli G, Charron JB, Yoon HJ, Chen H, He YJ, Xiong Y, Deng XW (2008) Characterization of *Arabidopsis* and rice DWD proteins and their roles as substrate receptors for CUL4-RING E3 ubiquitin ligases. *Plant Cell* **20:** 152–167

Lee J, Zhou P (2007) DCAFs, the missing link of the CUL4-DDB1 ubiquitin ligase. *Mol Cell* **26:** 775–780

Makarevich G, Leroy O, Akinci U, Schubert D, Clarenz O, Goodrich J, Grossniklaus U, Köhler C (2006) Different *Polycomb* group complexes regulate common target genes in *Arabidopsis*. *EMBO Rep* **7:** 947–952

Makarevich G, Villar CB, Erilova A, Köhler C (2008) Mechanism of *PHERES1* imprinting in *Arabidopsis*. *J Cell Sci* **121:** 906–912

Marrocco K, Bergdoll M, Achard P, Criqui MC, Genschik P (2010) Selective proteolysis sets the tempo of the cell cycle. *Curr Opin Plant Biol* **13:** 631–639

Molinier J, Lechner E, Dumbliauskas E, Genschik P (2008) Regulation and role of *Arabidopsis* CUL4-DDB1A-DDB2 in maintaining genome integrity upon UV stress. *PLoS Genet* **4:** e1000093

Mutskov V, Felsenfeld G (2004) Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9. *EMBO J* **23:** 138–149

Niessen HE, Demmers JA, Voncken JW (2009) Talking to chromatin: post-translational modulation of *Polycomb* group function. *Epigenetics Chromatin* **2:** 10

Ohad N, Margossian L, Hsu YC, Williams C, Repetti P, Fischer RL (1996) A mutation that allows endosperm development without fertilization. *Proc Natl Acad Sci USA* **93:** 5319–5324

Pien S, Grossniklaus U (2007) *Polycomb* and *trithorax* group proteins in *Arabidopsis*. *Biochim Biophys Acta* **1769:** 375–382

Ríos G, Lossow A, Hertel B, Breuer F, Schaefer S, Broich M, Kleinow T, Jásik J, Winter J, Ferrando A, Farrás R, Panicot M, Henriques R, Mariaux JB, Oberschall A, Molnár G, Berendzen K, Shukla V, Lafos M, Koncz Z *et al* (2002) Rapid identification of *Arabidopsis* insertion mutants by non-radioactive detection of T-DNA tagged genes. *Plant J* **32:** 243–253

Roodbarkelari F, Bramsiepe J, Weinl C, Marquardt S, Novák B, Jakoby MJ, Lechner E, Genschik P, Schnittger A (2010) Cullin 4-ring finger-ligase plays a key role in the control of endoreplication cycles in *Arabidopsis* trichomes. *Proc Natl Acad Sci USA* **107:** 15275–15280

Ruggieri R, Tanaka K, Nakafuku M, Kaziro Y, Toh-e A, Matsumoto K (1989) MSI1, a negative regulator of the RAS-cAMP pathway in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **86:** 8778–8782

Scheuermann JC, de Ayala Alonso AG, Oktaba K, Ly-Hartig N, McGinty RK, Fraterman S, Wilm M, Muir TW, Müller J (2010) Histone H2A deubiquitinase activity of the *Polycomb* repressive complex PR-DUB. *Nature* **465:** 243–247

Schroeder DF, Gahrtz M, Maxwell BB, Cook RK, Kan JM, Alonso JM, Ecker JR, Chory J (2002) De-etiolated1 and damaged DNA binding protein1 interact to regulate *Arabidopsis* photomorphogenesis. *Curr Biol* **12:** 1462–1472

Smalle J, Vierstra RD (2004) The ubiquitin 26S proteasome proteolytic pathway. *Annu Rev Plant Biol* **55:** 555–590

Stock JK, Giadrossi S, Casanova M, Brookes E, Vidal M, Koseki H, Brockdorff N, Fisher AG, Pombo A (2007) Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* **9:** 1428–1435

Stolpe T, Süsslin C, Marrocco K, Nick P, Kretsch T, Kircher S (2005) *In planta* analysis of protein-protein interactions related to light signaling by bimolecular fluorescence complementation. *Protoplasma* **226:** 137–146

Sugasawa K, Okuda Y, Saijo M, Nishi R, Matsuda N, Chu G, Mori T, Iwai S, Tanaka K, Tanaka K, Hanaoka F (2005) UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. *Cell* **121:** 387–400

Vielle-Calzada J-P, Thomas J, Spillane C, Coluccio A, Hoeppner MA, Grossniklaus U (1999) Maintenance of genomic imprinting at the *Arabidopsis MEDEA* locus requires zygotic *DDM1* activity. *Genes Dev* **13:** 2971–2982

Wang H, Wang L, Erdjument-Bromage H, Vidal M, Tempst P, Jones RS, Zhang Y (2004) Role of histone H2A ubiquitination in *Polycomb* silencing. *Nature* **431:** 873–878

Wang D, Tyson MD, Jackson SS, Yadegari R (2006a) Partially redundant functions of two SET-domain *Polycomb* group proteins in controlling initiation of seed development in *Arabidopsis*. *Proc Natl Acad Sci USA* **103:** 13244–13249

Wang H, Zhai L, Xu J, Joo HY, Jackson S, Erdjument-Bromage H, Tempst P, Xiong Y, Zhang Y (2006b) Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol Cell* **22:** 383–394

Xu L, Shen WH (2008) Polycomb silencing of KNOX genes confines shoot stem cell niches in *Arabidopsis*. *Curr Biol* **18:** 1966–1971

Zhou W, Zhu P, Wang J, Pascual G, Ohgi KA, Lozach J, Glass CK, Rosenfeld MG (2008) Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation. *Mol Cell* **29:** 69–80

6.2 Primer Sequences

Table 6-1. Diagnostic primers for the *dde2-2* mutation

primer name	primer sequence (5' to 3')
Pr_dde_F	GGTGTACAGAGTCAACATGCCAC
Pr_dde_R	AGGTACGAGAGGATACGGTAGC

Table 6-2. Primers for the T-DNA insertion lines genotyping

name	gene	primer sequence (5' to 3')
Pr_Salk_102417	*KRP4*	LP TGGTTAAAATTGAAACTGGCG
		RP CCTGGTAGTGGTTGTTCGTTC
Pr_Salk_130744	*KRP2*	LP GATTTATTGGAAAAGCCAGCC
		RP AGACTCTTCCTTAACCTGCGG
Pr_Sail_548_B03	*KRP6*	LP ACAAACCAACGAAATCACCG
		RP ATTCATCACCGGACTCTCATG
Pr_Gabi_841D12	*KRP7*	LP CGTTGAATTAATCAACGGCTC
		RP TCTTGGTACGAAGAACAGATGAAG
Pr_Salk_Lb1.3	-	ATTTTGCCGATTTCGGAAC
Pr_Sail_LB1	-	GCCTTTTCAGAAATGGATAAAT
Pr_Gabi_o8409 (pAC106)	-	ATATTGACCATCATACTCATTGC

Table 6-3. Tail-PCR primers

primer name	primer sequence (5' to 3')
Pr_Tail_1	TATAGCGCGCAAACTAGGATAAAT
Pr_Tail_2	TATCGCGCGCGGTGTCATCTA
Pr_Tail_3	CATCATACTCATTGCTGATCC
Pr_AD_1	NTCGASTWTSGWGTT
Pr_AD_2	NGTCGASWGANAWGAA

Table 6-4. Primers for Southern blot probes generation

primer name	primer sequence (5' to 3')
Pr_MJ1_probe_F	GCTTGGGTGGAGAGGCTATT
Pr_MJ1_probe_R	GTCAAGAAGGCGATAGAAGGCG
Pr_MJ2_probe_F	GAGACAAGCAGCGTCAACTTC
Pr_MJ2_probe_R	ACTTCAGCAGGTGGGTGTAGA

Table 6-5. Primers for *AtEASE*/min35S amplification from pWY-093.1 vector

primer name	primer sequence (5' to 3')
Pr_XbaI_EASE_F	AAAAATCTAGACTAAAACCAAAATCCAGGGGTAC
Pr_AscI_min35SEASE_R	AAAAGGCGCGCCCTCTCCAAATGAAATGAACTTCC

Table 6-6. Primers for *CDS* Gateway™ cassette

primer name	primer sequence (5' to 3')
Pr_attB1_CDKA;1_F	AAAAAGCAGGCT ATGGATCAGTACGAGAAAGT
Pr_attB2_CDKA;1_R	AGAAAGCTGGGT CTAAGGCATGCCTCCAAGA
Pr_attB1_CDKD;3_F	AAAAAGCAGGCT ATGCCGGAGCAGCCAAAGA
Pr_attB2_CDKD;3_R	AGAAAGCTGGGT TTACTGGAACTCAAGATCGA
Pr_attB1_CycA3;1_F	AAAAAGCAGGCT ATGGCCGACGAAAAAGAGAA
Pr_attB2_CycA3;1_R	AGAAAGCTGGGT CTAAATGTTGACATCTTCAAA
Pr_attB1_CycD2;1_F	AAAAAGCAGGCT ATGGCTGAGAATCTTGCTT
Pr_attB2_CycD2;1_R	AGAAAGCTGGGT TCATTGTTTTCTCCTCCTCT
Pr_attB1_CycD3;1_F	AAAAAGCAGGCT ATGGCGATTCGGAAGGAGGA
Pr_attB2_CycD3;1_R	AGAAAGCTGGGT TTATGGAGTGGCTACGATTG
Pr_attB1_CycH;1_F	AAAAAGCAGGCT ATGGCGGATTTTCAGACAT
Pr_attB2_CycH;1_R	AGAAAGCTGGGT TCAACCTATGGGTGGCGGT
Pr_attB1_DPb_F	AAAAAGCAGGCT ATGACAACTACTGGGTCTAAT
Pr_attB2_DPb_R	AGAAAGCTGGGT TCAATTCTCCGGCTTCATGCT
Pr_attB1_E2Fb_F	AAAAAGCAGGCT ATGTCTGAAGAAGTACCTCAA
Pr_attB2_E2Fb_R	AGAAAGCTGGGT TCAGCTACCTGTAGGTGATCT
Pr_attB1 adapter	GGGGACAAGTTTGTACAAAAAAGCAGGC
Pr_attB2 adapter	GGGGACCACTTTGTACAAGAAAGCTGGGT

Table 6-7. Primers for Artificial MicroRNA

gene	primer name	primer sequence (5' to 3')
MSI1	I_miR-s	gaTATCATCGTACTGACGAGGTTtctctcttttgtattcc
	II_miR-a	gaAACCTCGTCAGTACGATGATAtcaaagagaatcaatga
	III_miR*s	gaAAACTCGTCAGTAGGATGATTtcacaggtcgtgatatg
	IV_ miR*a	gaAATCATCCTACTGACGAGTTTtctacatatatattcct
RBR1	I_miR-s	gaTACTGTGTGAAATAAGAGCGTtctctcttttgtattcc
	II_miR-a	gaACGCTCTTATTTCACACAGTAtcaaagagaatcaatga
	III_miR*s	gaACACTCTTATTTCTCACAGTTtcacaggtcgtgatatg
	IV_ miR*a	gaAACTGTGAGAAATAAGAGTGTtctacatatatattcct

Table 6-8. Primers used for the *OlexA-TATA* fragment amplification in pLB71

primer name	**primer sequence (5' to 3')**
Pr_ *OlexA-TATA*/min35S_F	TCTAGAACTAGTGGATCCCCC
Pr_ *OlexA-TATA*/min35S_R	GTACTAGTGGTACCGGGCCCC

6.3 Result Tables

Table 6-9. X^2 test results. The 3: 1 segregation ratio was significant at P< 0.05. Lines showing the 3: 1 segregation ratio were marked in green.
n-number of counted seedlings, O-observed segregation, E-expected segregation

line		n	O		E		X^2
name	nr		green	white	green	white	
CycA3;1	1	12	11	1	9	3	1,78
	3	211	142	69	158,25	52,75	6,67
	6	151	113	38	113,25	37,75	0,0022
	8	60	50	10	45	15	2,22
	11	201	152	49	150,75	50,25	0,04
	22	86	60	26	64,5	21,5	0,97
	30	210	167	43	157,5	52,5	2,29
	31	197	151	46	147,75	49,25	0,96
CycD2;1	1	88	70	18	66	22	0,96
	3	243	185	58	182,25	60,75	0,16
	4	106	88	18	79,5	26,5	3,66
	5	98	78	20	73,5	24,5	1,11
	6	91	71	20	68,25	22,75	0,23
	8	198	147	51	148,5	49,5	0,06
CycD3;1	6	99	75	24	74,25	24,75	0,03
	8	64	46	18	48	16	0,33
	27	73	54	19	54,75	18,25	0,041
	28	76	57	19	57	19	0
	31	160	116	44	120	40	0,53
	33	63	45	18	47,25	15,75	0,43
	1a	111	86	25	83,25	27,75	0,36
	2a	60	51	9	45	15	3,20
	9a	119	89	30	89,25	29,75	0,003
	10a	75	61	14	56,25	18,75	1,60
CycH;1	2	121	98	23	90,75	30,25	0,58
	3	162	86	76	121,5	40,5	41,5
	8	84	70	14	63	21	3,11
	10	50	40	10	37,5	12,5	0,67
	12	23	17	6	17,25	5,75	0,015
	13	158	104	54	118,5	39,5	7,09
	14	119	94	24	89,25	29,75	1,48
	15	65	40	25	48,75	16,25	6,28
	16	65	50	15	48,75	16,25	0,13
CDKD;3	2	79	61	18	59,25	19,75	0,20
	3	116	83	33	87	29	0,73
	8	38	26	12	28,5	9,5	0,88
	9	39	32	7	29,25	9,75	0,09
	10	71	42	29	53,25	17,75	9,51
	11	118	89	29	88,5	29,5	0,01
	14	101	82	19	75,75	25,25	2,07
	15	21	20	1	15,75	5,25	4,60
	16	110	80	30	82,5	27,5	0,31
	17	10	9	1	7,5	2,5	1,20
DPb	1	168	132	36	126	42	1,15
	2	112	87	25	84	28	0,44
	3	139	98	41	104,25	34,75	1,51
DPb	5	88	67	21	66	22	0,06
	6	54	41	13	40,5	13,5	0,02
	7	98	72	26	73,5	24,5	0,12
	12	57	49	8	42,75	14,25	3,65
CDKA,1	2a	76	56	20	57	19	0,07
	9	132	92	40	99	33	2,00
	8a	154	116	38	115,5	38,5	0,0085
	9a	78	62	16	58,5	19,5	0,84
	10a	163	100	63	122,25	40,25	16,8
E2Fb	4	184	138	46	138	46	0
	6	224	168	56	168	56	0
	7	74	59	15	55,5	18,5	0,88
	12	138	96	42	103,5	34,5	2,17
	14	153	117	36	114,75	38,25	0,17

Table 6-10. The T-DNA position in the genome and sequences of insertion specific primers for lines overexpressing CDKD;3 (line 2), CycA3;1 (line 30), CycD2;1 (line 8), and CycH;1 (line 10).

line	insertion position	primer sequence 5'- 3'
pMJ1-CDKD;3_2	Chr. 2/ 9923933	F TGTACAACAACCAACCTAAACC R ACTCCTTATTAAACTGCTAGTCAT
pMJ1-CycA3;1_30	Chr. 1/ 25614889	F AACTCTTTCTGCTCCGTTTCT R TCAGCTTGGTCAGGAACATTAT
pMJ1-CycD2;1_8	Chr. 5/ 24667743	F CTGAAAATGAGCTAAACATGGC R AATTGAGTCGACGGAGGTTT
pMJ1-CycH;1_10	Chr. 4/ 13482915	F ACGGCTATGTTGTTTGTCTATG R ATAATGGATGCGAATAGACCGA

Table 6-11. Table of mutant lines overexpressing the G1/S phase genes the observed phenotype.

line genotype	generation/number of screened plants	phenotype
CDKA;1/+;CycA3;1/+ CDKA;1/+;CycD2;1/+ CDKA;1/+;CycD3;1/+ CDKA;1/+;CycH;1/+ CDKA;1/+;CDKD;3/+	F_1/ 8 mutant plants	first crosses:~50% embryo sac abortion; second crosses: normal embryo sacs; no developing embryos
CDKA;1/+;CDKD;3/+;CycD2;1/+ CDKA;1/+;CDKD;3/+;CycA3;1/+ CDKA;1/+;CDKD;3/+;CycH;1/+	F_1/ 8 mutant plants	normal embryo sacs; no developing embryos
CDKA;1/+;CycD2;1/+;CycH;1/+	F_1/ 8 mutant plants	37-53% aborted embryo sacs at functional megaspore stage
CDKA;1*;CDKD;3/+;*krp2*/*krp2*;*krp4*/*krp4*;*krp7*/*krp7*	F_2/ 1 mutant plant	4.5% multiple egg cell nuclei
CDKA;1*;CDKD;3/+;*krp2*/*krp2*;*krp4*/+;*krp6*/*krp6*;*krp7*/+	F_2/ 1 mutant plant	normal embryo sacs; no developing embryos
CycD2;1/+; *krp2*/*krp2*;*krp4*/+;*krp6*/*krp6*;*krp7*/*krp7*	F_2/ 1 mutant plant	embryo sac abortion at four or two nuclei stage

* There were no the T-DNA insertion specific primers for this line. To genotype this insertion, primers pr_AtEASE/min35S_F (Table 6-5) and pr_attB2_CDKA;1_R (Table 6-6) were used.

7 REFERENCES

Ach RA, Taranto P, Gruissem W (1995) A Conserved Family of WD-40 Proteins Binds to the Retinoblastoma Protein in both Plants in Animals. Plant Cell **9**: 1595-1606

Albertini E, Marconi G, Barcaccia G, Raggi L, Falcinelli M (2004) Isolation of Candidate Genes for Apomixis in *Poa pratensis* L. Plant Mol Biol **56**: 879-894

Albertini E, Marconi G, Reale L, Barcaccia G, Porceddu A et al (2005) SERK and APOSTART. Candidate Genes for Apomixis in *Poa pratensis.* Plant Physiol **138**: 2185-2199

Alvarez JP, Pekker I, Goldshmidt A, Blum E, Amsellem Z, Eshed Y (2006) Endogenous and Synthetic MicroRNAs Stimulate Simultaneous, Efficient, and Localized Regulation of Multiple Targets in Diverse Species. Plant Cell **18**: 1134-1151

Antoine AF, Dumas C Faure JE, Feijo JA, Rougier M (2001) Egg Activation in Flowering Plants. Sex Plant Reprod **14**: 21-26

Antoine AF, Faure JE, Cordeiro S, Dumas C, Rougier M, Feijo JA (2000) A Calcium Influx is Triggered and Propagates in the Zygote as a Eavefront During In Vitro Fertilization of Flowering Plants. Proc Natl Acad Sci USA **97**: 10643-10648

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J et al (2003) Genome-Wide Insertional Mutagenesis of *Arabidopsis thaliana.* Science **301(5641)**: 1849

Asker SE, Jerling L (1992) Apomixis in Plants. Boca Raton, FL: CRC Press

Bajon C, Horlow C, Motamayor JC, Sauvanet A, Robert D (1999) Megasporogenesis in *Arabidopsis thaliana* L. :an Ultrastructural Study. Sex Plant Reprod **12**, 99-109

Barcaccia G, Mazzucato A, Albertini E, Zethof J, Gerats A (1998) Inheritance of Parthenogenesis *inPoa pratensis* L. Auxin Test and AFLP Linkage Analysis Support Monogenic Control Theor Appl Genet **97**: 74-82

Baroux C, Blanvillain R, Betts H, Batoko H, Craft J, Martiniez A, Gallois P, Moore I (2005) Predictable Activation of Tissue-Specific Expression from a Single Gene Locus Using the pOp/LhG4 Transactivation System in Arabidopsis. Plant Biotechnology J 3: **91**-101

Barrell PJ, Conner RJ (2006) Versatile Minimal T-DNA Binary Vectors for Transgene Pyrimiding in Plants. Biotechniques **41**: 708-710

Bashaw EC, Hanna WW (1990) Reproductive Versatility in the Grasses. *In* GP Chapman, ed, Apomictic Reproduction, Cambridge UK, Cambridge University Press, Pp 100- 130

Berger F (1999) Endopserm Development. Curr Opin Plant Biol **2**: 28-32

Bernard P, Couturier M (1992) Cell Killing by the F Plasmid CcdB Protein Involeves Poisoning of DNA-Topoisomerase II Complexes. J Mol Biol **226**: 735-745

Bicknell RA, Bicknell KB (1999) Who Will Benefit From Apomixis? Biotechnol Dev Monit **37**: 17-20

Bicknell RA, Borst NK Koltunow AM (2000) Monogenic Inheritance of Apomixis in Two *Hieracium* Species with Distinct Developmental Mechanisms. Heredity **84**: 228-237

Bicknell RA, Koltunow AM (2004) Understanding Apomixis: Recent Advances and Remaining Conundrums. Plant Cell **16**: S228-245

Boavida LC, Vieira AM, Becker JD, Feijo JA (2005) Gametophyte Interactions and Sexual Reproduction: How Plants Make a Zygote. Int J Dev Biol **49**: 615-632

Boisson-Dernier A, Roy S, Kritsas K, Grobei MA, Jaciubek M, Schroeder JI, Grossniklaus U (2009) Dissruption of the Pollen-Expressed FERONIA Homologs ANXUR1 and ANXUR2 Triggers Pollen Tube Discharge. Development **136**: 3279-3288

Boniotti MB, Gutierrez C (2001) A Cell-Cycle Regulated Kinase Activity Phosphorylates Plant Retinoblastoma Protein and Contains, in Arabidopsis, a CDKA/Cyclin D Complex. Plant J **28**: 341-350

Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Liu CM, van Lammern AA, Miki BL, Custers JB, van Lookeren Campagne MM (2002) Ectopic Expression of BABY BOOM Trigger a Conversion from Vegetative to Embryonic Growth. Plant Cell **14**: 1737-1749

Brand L (2007) Development of a Stringent and Cell-Type Specific Inducible Gene Expression System to Engineer Elements of Apomixis in *Arabidopsis thaliana*. PhD Thesis, University of Zürich

Brand L, Hörler M, Nüesch E, Vassalli S, Barrell P, Yang W, Jefferson RA, Grossniklaus U, Curtis MD (2006) A Versatile and Reliable Two Component System for Tissue-Specific Gene Induction in *Arabidopsis*. Plant Physiol **141**: 1194-1204

Brendel V, Kleffe J, Carle-Urioste JC, Walbot V (1998) Prediction of Splice Sites in Plant Pre-mRNA from Sequence Properties. J Mol Biol **276**: 85-104

Brukhin VB, Jaciubek M, Bolanos-Carpio A, Kuzmina V, Grossniklaus U (2011) Female Gametophytic Mutants of Arabidopsis Thaliana Identified in a Gene Trap Insetional Mutagenesis Screen. Int J Dev Biol **55**: 73-84

Calderini O, Chang S, de Jong H, Busti A, Paolocci F et al (2006) Molecular Cytogenetics and DNA Sequence Analysis of an Apomixis-Linked BAC in Paspalum simplex Reveal a Non Pericentromere Location and Partial Microcolonearity with Rice. Theor App Genet **112**: 1179-1191

Clough SJ, Bent AF (1998) Floral Dip: A Simplified Method for Agrobacterium Mediated Transformation of Arabidopsis thaliana. Plant J **16**: 735-753

Capron A, Gourgues M, Neiva LS, Faure JE, Berger F, Pagnussat G, KKrishnan A, Alvarez-Mejia C, Vielle-Calzada JP, Lee YR (2008) Maternal Control of Male-Gamete Delivery in *Arabidopsis* Involves a Putative GPI-Anchored Protein Encoded by the LORELEI gene. Plant Cell **20**: 3038-3049

Carman JG (1997) Asynchronous Expression of Duplicate Genes in Angiosperms May Cause Apomixis, Bispory, Tetraspory and Polyembryony. Biol J Linn Soc **61**: 51-94

Carman JG(2007) Do Duplicate Gene Cause Apomixis. In E. Hörandl, U Grossniklaus, van Dijk PJ, Sharbel TF, eds, Apomixis: Evolution, Mechanisms and Perspectives. ARG Gantner Verlag, Rugell, Liechtenstein, pp 63-91

Catanach AS, Erasmuson SK, Podvinsky E, Jordan BR, Bicknell R (2006) Colloquium paper: Deletion Mapping of Genetic Regions Associated with Apomixis in *Hieracium*. Proc Natl Acad Sci USA 103: 18650-55

Chaudhury AM, Koltunow AM, Payne T, Luo M, Tucker MR, Dennis ES, Peacock WJ (2001) Control of Early Seed Development. Annu Rev Cell Dev Biol **17**: 677-699

Chaudhury AM, Ming L, Miller C, Craig S, Dennis ES, Peacock WJ (1997) Fertilization-Independent Seed Development in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **94**: 4223-4228

Chen L, Miyazaki C, Kojima, Saito A, Adachi T (1999) Isolation and Characterization of a Gene Expressed During Early Embryo Sac Development in an Apomictic Guinea Grass (*Pannicum maximum*) J Plant Physiol **154**: 55-62

Chen Y-H, Li H-J, Shi DQ, Yuan L, Liu J, Sreenivasan R, Ramarmurthy B, Grossniklaus U, Yang W-C (2007) The Central Cell Plays a Critical Role in Pollen Tube Guidance in *Arabidopsis.* The Plant Cell **19**: 3563-3577

Christensen CA, King EJ, Jordan JR, Drews GN (1997) Megagametogenesis in Arabidopsis Wild-Type and the Gf Mutant. Sex Plant Reprod **10**, 49-64

Christensen CA, Gorsich SW, Brown RH, Jones LG, Brown J, Shaw JM, Drews GN (2002) Mitochondrial GFA2 is Required for Synergid Cell Death in *Arabidopsis*. Plant Cell **14**: 2215-2232

Cockcroft CE, den Boer BGW, Healy JMS, Murray JAH (2000) Cyclin D Control of Growth Rate in Plants. Nature **405**: 575-579

Conner JA, Goel S, Gunawan G, Cordonnier-Pratt MM, Johnson VE, Liang C, Wang H, Pratt LH, Mullet JE, DeBarry J, Yang L, Bennetzen JL, Klein PE, Ozias-Akins P (2008) Sequence Analysis of Bacterial Artificial Chromosome Clones From Apospory-Specific Genomic Region of Pennisetum and Cenchrus. Plant Physiol. **147(3)**: 1396-411

Craft J, Samalova M, Baroux C, Townley H, Martinez A, Jepson I, Tsiantis M, Moore I (2005) New pOp/LhG4 Vectors for Stringent Glucocorticoid-Dependent Transgene Expression in Arabidopsis. Plant J **41**:899-918

Curtis MD, Grossniklaus U (2003) A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. Plant Physiol **133**: 462-469

Curtis MD, Grossniklaus U (2007) Amphimixis and Apomixis: Two Sides of the Same Coin! In E. Hörandl, U Grossniklaus, van Dijk PJ, Sharbel TF, eds, Apomixis: Evolution, Mechanisms and Perspectives. ARG Gantner Verlag, Rugell, Liechtenstein, pp 137-158

del Pozzo JC, Boniotti MB, Gutierrez C (2002) Arabidopsis E2Fc Functions in Cell Division and is Degraded by the Ubiquitin-SCF^{AtSKP2} Pathway in Response to Light. Plant Cell **14**: 3057-3071

den Boer BGW, Murray JAH (2000) Control of Plant Growth and Development Through Manipulation of Cell Cycle Genes. Curr Opin Biotechnol **11**: 138-145

Deveaux Y, Peaucelle A, Roberts GR, Coen E, Simon R, Mizukami Y, Traas J, Murray JA, Doonan JH, Laufs P (2003) The Ethanol Switch: A Tool for Tissue-Specific Gene Induction During Plant Development. Plant J **36**: 918-930

De Veylder L, Beeckman T, Beemster GT, Krols L, Terras F, Landreiu I, van der Schueren E, Maes S, Naudts M, Inze D (2001) Functional Analysis of Cyclin-Dependent Kinase Inhibitors of Arabidopsis. Plant Cell **13**: 1653-1668

Drews GN, Koltunow AMG (2011) The Female Gametophyte. The Arabidopsis Book, American Society of Plant Biologists

Dumbliauskas E, Lechner E, Jaciubek M, Berr A, Pazhouhandeh M, Alioua M, Cognat V, Brukhin V, Koncz C, Grossniklaus U, Molinier J, Genschik P (2011) The Arabidopsis CUL4-DDB1 Complex Interacts with MSI1 and is Required to Maintain MEDEA Parental Imprinting. EMBO J **30**: 731-743

Durfee T, Feiler HS, Gruissem W (2000) Retinoblastoma-Related Proteins in Plants: Homologues or Orthologues for their Metazoan Counterparts. Plant Mol Biol **43**: 635-642

Ebel C, Mariconti L, Gruissem W (2004) Plant Retinoblastoma Homologues Control Nuclear Proliferation in the Female Gametophyte. Nature **429**: 776-780

Ebina M, Nakagawa H, Yamamoto T, Araya H, Tsuruta S et al. (2005) Co-segregation of AFLP and RAPD Markers to Apospory in Guineagrass (*Panicum maximum* Jacq.). Grassland Sci **51**: 71-78

d`Erfurth I, Jolivet S, Froger N, Catrice O, Novatchkova M, Mercier R (2009) Turning Meiosis into Mitosis. PLoS Biol **7**(6): e1000124

Escobar-Restrepo JM, Huck N, Kessler S, Gagliardini V, Gheyselinck J, Yang WC, Grossniklaus U (2007) The FERONIA Receptor-Like Kinase Mediates Male-Female Interactions During Pollen Tube Reception. Science **317**: 656-660

Faure JE, Rotman N, Fortune P, Dumas C (2002) Fertilization in Arabidopsis thaliana Wild Type: Developmental Stages and Time Course. Plant J **30**: 481-488

Francis D (2007) The Plant Cell Cycle – 15 Years On. New Phytologist **174**: 261-278

Friedman WE (1998) The Evolution of Double Fertilization and Endopserm an ”Historical” Perspective. Sex Plant Rep. **11**: 6-16

Garcia-Aguilar M, Michaud C, Leblanc O, Grimanelli D (2010) Inactivation of a DNA Methylation Pathway in Maize Reproductive Organs Results in Apomixis-Like Phenotypes. Plant Cell **22**: 3249-3267

Genschik P, Criqui MC, Parmentier Y, Derevier A, Fleck J (1998) Cell-Cycle Dependent Proteolysis in Plants: Identification of the Destruction Box Pathway and Metaphase Arrest produced by the Protesase Inhibitor MG132. Plant Cell **10**: 2063-2075

Gifford EM, Foster AS (1989) Morphology and Evolution of Vascular Plants. (New York: W.H. Freeman and Company)

Grimanelli D (2012) Epigenetic Regulation of Reproductive Development and the Emergence of Apomixis in Angiosperms. Curr Opin Plant Biol **15**: 57-62

Grimanelli D, Hernandez M, Perotti E, Savidan Y (1997) Dasage Effect in the Endosperm of the Diplosporous Apomictic *Tripsacum* (Poaceae). Sex Plant Reprod **10**: 279-282

Grimanelli D, Leblanc O, Espinoza E Perotti E, Gonzales de Leon D, Savidan Y (1998) Mapping Diplosporous Apomixis in Tetraploid *Tripsacum*: One gene or Several Genes? Heredity **80**: 33-39

Grimanelli D, Leblanc O, Perotti E, Grossniklaus U (2001) Developmental Genetics of Gametophytic Apomixis. Trends Genet **17**: 597-604

Gross-Hardt R, Lenhard M, Laux T (2002) WUSCHEL Signalling Functions in Interregional Communication During Arabidopsis Ovule Development. Genes Dev **16**: 1129-1138

Grossniklaus U, Moore JM, Gagliano WB (1998) Molecular and Genetic Approaches to Understanding and Engineering Apomixis: Arabidopsis as a Powerful Tool. In SS Virmani, EA Siddiq, K Muralidharan, eds, Proceedings of the 3rd International Symposium on Hybrid Rice. In Advances in Hybrid Rice Technology, International Rice Research Institute, Minila, Philippines, pp 187-211

Grossniklaus U (2001) From Sexuality to Apomixis: Molecular and Genetic Approaches. In Flowering of Apomixis: From Mechanisms to Genetic Engineering, Y Savidan, JG Carman, T DresselHaus, eds, Mexico: CIMMYT, IRD, European Commission DG VI, pp 168-211

Grossniklaus U, Nogler GA, van Dijk PJ (2001) How to Avoid Sex: The Control of Gametophytic Apomixis. The Plant Cell **13**: 1477

Grossniklaus U, Vielle-Calzada JP, Hoepner MA, Gagliano WB (1998) Maternal Control of Embryogenesis by MEDEA, a Polycomb Group Gene in *Arabidopsis.* Science **280**: 446-450

Gu Z, Steinmetz LM, Gu X, Scharfe C, Davis RW, Li WH (2003) Role of Duplicated Genes in Genetic Robustness Against Null Mutations. Nature **421**: 63-66

Guiton EA, Berger F (2005) Loss of Function of MULTICOPY SUPPRESSOR OF IRA 1 Produces nonviable parthenogenetic embryos in Arabidopsis. Curr Biol **15**: 750-754

Guiton EA, Page DR, Chambrier P, Lionnet C, Faure JE, Grossniklaus U, Berger F (2004) Identification of New Members of Fertilization Independent Seed Polycomb

Group Pathway Involved in the Control of Seed Development in *Arabidopsis thaliana*. Development **131**: 2971-2981

Gustine DL, Sherwood RT, Huff DR (1997) Apospory-Linked Molecular Markers in Buffelgrass. Crop Sci **37**: 947-951

Gutierrez C (2009) The Arabidopsis Cell Division Cycle. The Arabidopsis Book. American Society of Plant Biologists **7**: e0120

Gutierrez C, Ramirez-Parra E, Castellano MM, del Pozo JC (2002) G(1) to S Transition: More Than a Cell Cycle Engine Switch. Curr Opin Plant Biol **5**: 480-486

Hagberg A, Hagberg G (1980) High Frequency of Spontaneous Haploids in the Progeny of an Induced Mutation in Barley. Hereditas **93**: 341-343

Haig D. Westoby M. (1991) Genomic Imprinting in Endosperm: its Effects on Seed Development in Crosses Between Species and Between Different Ploidies of the Same Species, and its Implications for the Evolution of Apomixis. Philos Trans R Soc Lond B Biol Sci **333**: 1-13.

Harbour JW, Dean DC (2000) The Rb/E2F Pathway: Expanding Roles and Emerging Paradigms. Genes Dev **14**: 2393-2409

Healy JMS, Menges M, Doonan JH, Murray JAH (2001) The Arabidopsis D-Type Cyclind CycD2;1 and CycD3;1 both Interact in Vivo with the PISTAIRE Cyclin-Dependent Kinase Cdc2a but are Differentially Controlled. J Biol Chem **276**: 7041-7047

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt EDL, Boutilier K, Grossniklaus U, de Vries SC (2001) The *Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* Gene is Expressed in Developing Ovules and Embryos and Enhances Embryonic Competence in Culture. Plant Physiol **127**: 803-816

Hemerly A, de Almeida Engler J, Bergounioux C, Van Montagu M, Engler G, **Inze D, Ferreira P** (1995) Dominant Negative Mutants of the Cdc2 Kinase Uncouple Cell Division from Iterative Plant Development. EMBO J **14**: 3925-3936

Higashiyama T, Hamamura Y (2008) Gametophytic Pollen Tube Guidance. Sex Plant Rep **21**: 17-26

Higashiyama T, Kuroiwa H, Kuroiwa T (2003) Pollen-Tube Guidance: Beacons from the Female Gametophyte. Curr Opin Plant Biol **6**: 36-41

Hirano H, Shinmyo A, Sekine M (2011) Both Negative and Positive G1 Cell Cycle Regulators Undergo Proteasome-Dependent degradation During Sucrose Starvation in Arabidopsis. Plant Signal Beh **6**: 1394-1396

Hsieh TF, Hakim O, Ohad N, Fischer RL (2003) From Flour to Flower: How Polycomb Group Proteins Influence Multiple Aspects of Plant Development. Trends Plant Sci **8(9)**: 439-445

Huang B-Q, Russel SD (1992) Female Germ Unit: Organization, Isolation, and Function. Int Rev Cytol **140**, 233-292

Hülskamp M, Kopczak SD, Horejsi TF, Kihl BK, Pruitt RE (1995) Identification of Genes Required for Pollen-Stigma Recognition in *Arabidopsis thaliana*. Plant J **8**:703-714

Inzé D, De Veylder L (2006) Cell Cycle Regulation in Plant Development. Annu Rev Genet **40**: 77-105

Jauhar PP (2006) Spontaneous Haploids in Durum Wheat: Their Cytogenetic Characterization. Euphytica **148**: 341-344

Jefferson RA (1994) Apomixis a Social Revolution For Agriculture? Biotechnol Dev Monit **19**: 14-16

Johnson MA, Preuss D (2002) Plotting a Course: Multiple Signals Guide Pollen Tubes to their Targets. Dev Cell **2**: 273-281

Johnston AJ, Matveeva E, Kirioukhova O, GGrossniklaus U, Gruissem W (2008) A Dynamic Reciprocal RBR-PRC2 Regulatory Circuit Controls Arabidopsis Gametophyte Development. Curr Biol **18**: 1680-1686

Johnston AJ, Meier P, Gheyselinck J, Wuest SE, Federer M, Schlagenhauf E, Becker JD, Grossniklaus U (2007) Genetic Subtraction Profiling Identifies Genes Essential for Arabidopsis Reproduction and Reveals Interaction Between the Female Gametophyte and the Maternal Sporophyte. Genome Biol **8**: R204

Joubes J, Chevalier C, Dudits D, Heberle-Bors E, Inze D (2000) CDK-Related Protein Kinases in Plants. Plant Mol Biol **43**: 607-620

Jullien PE, Mosquna A,Ingouff M, Sakata T, Ohad N, Berger F (2008) Retinoblastoma and Ist Binding Partner MSI1 Control Imprinting in Arabidopsis. PLoS Biol **6**: e194

Kantama L (2005) Chromosome Studies and Genetic Analysis of Natural and Synthetic Apomictic Model Species. University of Wageningen.

Kaushal P, Malaviya DR, Roy AK, Pathak S, Agrawal A, Khare A, Siddiqui SA (2008) Reproductive Pathways of Seed Development in Apomictic Guinea Grass (*Panicum maximum* Jacq.) Reveal Uncoupling of Apomixis Component. Euphytica **164**: 82-91

Kermicle J (1969) Androgenesis Conditioned by a Mutation in Maize. Science **116**: 1422-1424

Kessler SA, Shimosato-Asano H, Keinath NF, Wuest SE, Ingram G, Panstruga R, Grossniklaus U (2010) Conserved Molecular Components For Pollen Tube Reception and Fungal Invasion. Science, **330**: 968-971

Kimber G, Riley H (1963) Haploid Angiosperms. Bot Rev **29**: 480-531

Kiyosue T, Ohad N, Yadegari R, Hannon M, Dinneny J, Wells D, Katz A, Margossian L, Harada JJ, Goldberg RB, Fisher Rl (1999) Control of Fertilization-Independent Endosperm Development by the MEDEA Polycomb Gene in Arabidopsis. Proc Natl Acad Sci USA **96**: 4186-4191

Köhler K, Henning L, Bouveret R, Gheyselinck J, Grossniklaus U, Gruissem W (2003) Arabidopsis MSI1 is a Component of the MEA/FIE Polycomb Group Complex and Required for Seed Development. EMBO J **22**: 4804-4814

Kojima A, Nagato Y (1997) Discovery of Highly Apomictic and Highly Amphimictic Dihaploids in *Allium tuberosum*. Sex. Plant Reprod. **10**: 8-12

Koltunow AM (1993) Apomixis: Embryo Sac and Embryo Formed without Meiosis or Fertilization in ovules. Plant Cell **5**:1425-1437

Koltunow AM, Bicknell RA, ChaudhuryAM (1995) Apomixis: Molecular Strategies for the Generation of Genetically Identical Seeds without Fertilization. Plant Physiol **108**: 1345-1352

Koltunow AM, Grossniklaus U (2003) Apomixis: A Developmental Perspective. Annu Rev Plant Biol **54**: 547-574

Koltunow AM, Johnson SD, Bicnell RA (2000) Apomixis is Not Developmentally Conserved in Related, Genetically Characterized *Hieracium* Plants of Varying Ploidy. Sex. Plant Reprod. **12**: 253-266

Koltunow AM, Johnson SD, Okada T (2011) Apomixis in Hawkweed: Mendel`s Experimantal Nemesis. J Exp Bot **62 (5)**: 1699-1707

Koncz C. Schell J (1986) The Promoter of TL-DNA Gene 5 Controls Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of *Agrobacterium* Binary Vector. Molecular and General Genetics **204**: 383-396

Köszegi D, Johnston AJ, Rutten T, Czihal A, Altschmied L, Kumlehn J, Wuest SE, Kirioukhova O, Gheyselinck J, Grossniklaus U, Bäumlein H (2011) Mambers of the RKD Transcription Factor Family Induce An Egg Cell-Like Gene Expression Program. Plant J **67**: 280-291

Khraiwesh B, Ossowski S, Weigel D, Reski R, Frank W (2008) Specific Gene Silencing by Artificial MicroRNAs in Physcomitrella patens: An Alternative to Targeted Gene Knockouts. Plant Physiol **148**: 684-693

Krysan PJ, Young JC, Sussman MR (1999) T-DNA as an Insertional Mutagen in Arabidopsis. Plant Cell **11**: 2283-2290

Kumlehn J, Kirik V, Czihal A, Altschmied L, Matzk F, Lorz H, Baumlein H (2001) Parthenogenetic Egg Cell of Wheat: Cellular and Molecular Studies. Sex Plant Reprod **14**: 239-243

Labombarda P, Busti A, Caceres ME, Pupilli F, Acioni S (2002) An AFLP Marker Tightly Linked to Apomixis Reveals Hemizygosity in a Portion of the Apomixis-Controlling Locus in *Paspalum simplex*. Genome **45**: 513-519

Laux T, Mayer KF, Berger J, Jurgens GG (1996) The WUSCHEL Gene is Required for Shoot and Floral Meristem Integrity in Arabidopsis. Development **122**: 87-96

Laybourn PJ, Kadonaga JT (1992) Threshold Phenomena and Long-Distance Activation of Transcription by RNA Polymerase II. Science **257**: 1682-1685

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient Isolation and Mapping of Arabidopsis thaliana T-DNA Insert Junction by Thermal Asymmetric Interlaced PCR. Plant J **8**: 457-463

Lee HS, Chen ZL (2001) Protein-Codding Genes Are Epigenetically Regulated in Arabidopsis Polyploids. Proc Natl Acad Sci USA **98**: 6753-6758

Lin BY (1984) Association of Endosperm Reduction with Parental Imprinting in Maize. Genetics **107**: 103-115

Lord EM, Russell SD (2002) The Mechanisms of Pollination and Fertilization in Plants. Annu Rev Cell Dev Biol **18**: 81-105

Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW, Yamagishi K, Fischer RL, GGoldberg RB, Harada JJ (1998) Arabidopsis LEAFY COTYLEDON 1 is Sufficient to Induce Embryo Development in Vegetative Cells. Cell **93**: 1195-1205

Lui H, Wang H, Delong C, Fowke LC, Crosby WL, Fobert PR (2000) The Arabidopsis Cdc2a-Interacting Protein ICK2 is Structurally Related to ICK1 and is a Potent Inhibitor of Cyclin-Dependent Kinase Activity in Vitro. Plant J **21**: 379-385

Luo M, Bilodeau P, Dennis ES, Peacock WJ, Chaudhury A (2000) Expression Parent-of-Origin Effects for FIS2, MEA, and FIE in the Endosperm and Embryo of Developing of *Arabidopsis* Seeds. Proc Natl Acad Sci USA **97**: 10637-10642

Luo M, Bilodeau P, Koltunow AM, Dennis ES, Peacock WJ, Chaudhury A (1999) Genes Controlling Fertilization-Independent Seed Development in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **96**: 296-301

Maheshwari P. (1950) An introduction to the Embryology of Angiosperms. (New York: McGraw-Hill Publications)

Marimuthu MP, Jolivet S, Ravi M, Pereira L, Davda JN, Cromer L, Wang L, Nogue F, ChanSW, Siddiqi L, Mercier R (2011) Synthetic Clonal Reproduction Through Seeds. Science **331**: 876

Matsuhara S, Jingu F, Takahashi T, Komeda Y (2000) Heat-Shock Tagging: a Simple Method for Expression and Isolation of Plant Genome DNA Flanked by T-DNA Insertions. Plant J **22**: 79-86

Matzk F, Prodanovic S, Bäumlein H, Schubert I (2005) The Inheritance of Apomixis in *Poa pratensis* Confirms a Five Locus Model with Differences in Gene Expressivity and Penetrance. The Plant Cell **17**: 13-24

Mayer KF, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998) Role of WUSCHEL in Regulating Stem Cell Fate in the *Arabidopsis* Shoot Meristem. Cell **95**: 805-815

McCormic S (1993) Male Gametophyte Development. Plant Cell 5, 1265-1275

McCormic S (2004) Control of Male Gametophyte Development. Plant Cell 16, S142-S153

McElver JJ, Tzafrir I, Aux G, Rogers R, Ashby C, Smith K, Thomas C, Schetter A, Zhou Q et al (2001) Insertional Mutagenesis of Genes Required for Seed Development in *Arabidopsis thaliana*. Genetics **159**: 1751-1763

Menges M, de Jager SM, Gruissem W, Murray JA (2005) Global Analysis of the Core Cell Cycle Regulatores of Arabidopsis Identifies Novel Genes, Reveals Multiple and Highly Specific Profiles of Expression and Provides a Coherent Model for Plant Cell Cycle Control. Plant J **41**: 546-566

Menges M, Samland AK, Planchais S, Murray JAH (2006) The D-Type Cyclin CYCD3;1 is Limiting for the G1-to-S Phase Transition in Arabidopsis. Plant Cell 18: 893-906

Mercier R, Vezon D, Bullier E, Montamayor JC, Sellier A, Lefevre F, Pelletier G, Horlow C (2001) SWITCH1 (SWI1): a Novel Protein Required for the Establishment of Sister Chromatid Cohesion and for Bivalent Formation at Meiosis. Genes Dev **15**: 1859-1871

Miyazaki S, Ito M (2006) Calcium Signals for Egg Activation in Mammals. J Pharmacol Sci **100**: 545-552

Miyazaki S, Murata T, Sakurai-Ozato N, Kubo M, Demura T, Fukuda H, Hasebe M (2009), Curr Biol **19**: 1327-1331

Mogie M (1992) The Evolution of Asexual Reproduction in Plants. Chapman and Hall, New York

Murray JAH, Doonan J, Riou-Khamlichi C, Maijer M, Oakenfull EA (2001) G1 Cyclins, Cytokinins and the Regulation of the G1/S Transition. In Francis D, ed, The Plant Cell Cycle and Its Interfaces. CRC Press, Sheffield, pp19-41

Naumova TN, Osadtchiy J, Sharma VK, Dijkhuis P, Ramulu KS (1999) Apomixis in Plants: Structural and Functional Aspects of Diplospory in *Poa nemoralis* and *P. palustris* . Protoplasma **208**: 186-195

Naumova TN, van der Laak J, Osadtchiy J, Matzak F, Kravtchenko A, Bergervoet J, Ramulu KS, Boutilier K (2001) Reproductive Development in Apomictic Populations of *Arabis holboellii* (Brassicaceae). Sex Plant Reprod **14**: 195-200

Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH (2006) Expression of Artificial MicroRNAs in Transgenic *Arabidopsis thaliana* Confers Virus Resistance. Nat Biotachnol **24**: 1420-1428

Nogler GA (1984a) Gametophytic Apomixis. *In* BM Johri, ed, Embryology of Angiosperms, Springer Verlag, Berlin Germany

Nogler GA (1984b) Genetics of Apospory in Apomictic *Ranunculus auricomus*. V Conclusion Bot Helv **94**: 411-422

Nogler GA (1986) Genetics of Apomictic Reproduction. Bot Ital **120**: 49-52

Noyes RD (2006) Apomixis via Recombination of Genome Regions for Apomeiosis (Diplospory) and Parthenogenesis in Erigeron (Daisy fleabane, Asteraceae). Sex Plant Reprod **19**: 7-18

Noyes RD, Baker R, Mai B (2007) Mendelian Segregation for Two Factor Apomixis in Erigeron annuus (Asteraceae). Heredity **98**: 92-98

Noyes RD, Riesberg LH (2000) Two Independent Loci Control Agamospermy (Apomixis) in the Triploid Flowering Plant *Erigeron annuus.* Genetics **155**: 379-390

Noyes RD (2005) Inheritance of Apomeiosis (Diplospory) in Fleabanes (*Erigeron*, Asteraceae). Heredity **94**: 193-198

Odell JT, Nagy F, Chua NH (1985) Identification of DNA Sequences Required for Activity of the Cauliflower mosaic virus 35S promoter. Nature **313**: 810-812

Ohad N, Margossian L, Hsu YC, Williams C, Repetti P, Fisher RL (1996) A Mutation that Allows Endosperm Development without Fertilization. Proc Natl Acad Sci USA **93**: 5319-5324

Ohad N, Yadegari R, Margossian L, Hannon M, Michaeli D, Harada JJ, Goldberg RB, Fischer RL (1999) Mutations in FIE, a WD Polycomb Group Gene, Allow Endosperm Development without Fertilization. Plant Cell **11**: 407-415

Olmedo-Monfil V, Duran-Figueroa N, Arteaga-Vazquez M, Demesa-Arevalo E, Autran D, Grimanelli D, Slotkin RK, Martienssen RA, Vielle-Calzada JP (2010) Control of the Female Gamete Formation by a Small RNA Pathway in *Arabidopsis*. Nature **464**: 628-632

Ossowski S, Schwab R, Weigel D (2008) Gene Silencing in Plants Using Artificial MicroRNAs and Other Small RNAs. Plant J **53**: 674-690

Ozias-Akins P, Roche D, Hanna WW (1998) Tight Clustering and Hemizyosity of Apomixis-Linked Molecular Markers in *Pennisetum squamulatum* Implies Genetic Control of Apospory by a Divergent Locus which May Have No Allelic Form in Sexual Genotypes. Prot Natl Acad Sci USA **95**: 5127-5132

Ozias-Akins P, Van Dijk PJ (2007) Mendelian Genetics of Apomixis. Annu Rev Genet **41**: 509-37

Pessino SC, Espinoza F, Martinez EJ, Ortiz JPA, Valle EM, Quarin CL (2001) Isolation cDNA Clones Differentially Expressed in Flowers of Apomictic and Sexual *Paspalum notatum.* Hereditas **134**: 35-42

Pessino SC, Evans C, Ortiz JPA, Armstead I, do Valle CB, Hayward MD (1998) A Genetic Map of the Apospory-Region in *Bracharia* Hybrids: Identification of Two Markers Closely Associated with the Trait. Hereditas **134**: 35-42

Pien S, Wyrzykowska J, McQueen-Mason S, Smart C, Fleming A (2001) Local Expression of Expansin Induces the Entire Process of Leaf Development and Modifies Leaf Shape. Proc Natl Acad Sci USA **98**: 11812-11817

Pillai RS, Bhattacharyya SN, Filipowicz W (2007) Repression of Protein Synthesis by miRNAs: How Many Mechanisms? Trends Cell Biol **17**: 118-126

Planchais S, Samland AK, Murray JAH (2004) Different Stability of Arabidopsis D-Type Cyclins: CYCD3;1 is a Highly Unstable Protein Degraded by a Proteasome-Dependent Mechanism. Plant J **38**: 616-625

Polegri L, Calderini O, Arcioni S, Pupilli F (2010) Specific Expression of Apomixis-Linked Alleles Revealed by Comparative Transcriptomic Analysis of Sexual and Apomictic *Paspalum simplex* Morong Flowers. J Exp Biol **61**: 1869-1883

Pupilli F, Labombarda P, Caceres ME, Quarin CL, Arcioni S (2001) The Chromosome Segment Related to Apomixis in *Paspalum simplex* is homoeologous to the Telomeric Region of the Long Arm of the Rice Chromosome 12. Mol Breed **8**: 53-61

Quarin CL (1999) Effects of Pollen Source and Pollen Ploidy on Endosperm Formation and Seed Set in Pseudogamous Apomictc Paspalum *notatum.* Sex Plant Reprod **11**: 331-583

Quarin CL, Espinoza F, Martinez EJ, Pessino SC, Bovo OA (2001) A Rise of Ploidy Level Induces the Expression of Apomixis in *Paspalum notatum*. Sex Plant Reprod **13**: 243-249

Qi R, John PC (2007) Expression of Genomic AtCycD2;1 in Arabidopsis Induces Cell Division at Smaller Cell Sizes: Implications for the Control of Plant Growth. Plant Physiol **144**: 1587-1597

Raghavan V (2003) Some Reflections on Double Fertilization, from its Discovery to the Present. New Physiologist, **159**: 565-583

Ramirez-Parra E, Gutierrez C (2000) Characterization of Wheat DP, a Heterodimerization Partner of the Plant E2F Transcription Factor which Stimulates E2F DNA Binding. FEBS Lett **486**: 73-78

Ramirez-Parra E, Fründt C, Gutierrez C (2003) A Genome-Wide Identification of E2F-Regulated Genes in Arabidpsis. Plant J **33**: 801-811

Ravi M, Chan WLS (2010) Haploid Plants Produced by Centromere-Mediated Genome Elimination. Nature **464**: 615-619

Ravi M, Marimuthu MPA, Siddiqi I (2008) Gamete Formation without Meiosis. Nature **451**: 1121-1125

Rhoades MM, Dempsey E (1966) Induction of Chromosome Doubling by the Elongate Gene in Maize. Genetics **54**: 505-522

Roche D, Hanna WW, Ozias-Akins P (2001) Is Supernumerary Chromatin Involved in Gametophytic Apomixis of Polyploid Plants? Sex Plant Reprod **13**: 343-349

Rodrigues JCM, Okada T, Johnoson SD, Koltunow AM (2010) A MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) Homologue is not Associated with the Switch to Autonomous Seed Development in Apomictic (Asexual) *Hieracium* Plants Plant Sci **179(6)**: 590-597

Rodrigues JCM, Tucker MR, Johnson SD, Hrmova M, Koltunow AM (2008) Sexual and Apomictic Seed Formation In *Hieracium* Requires Plant Polycomb- Group Gene *FERTILIZATION INDIPENDENT ENDOPSERM*. Plant Cell **20(9)**: 2372-2386

Rossi V, Varotto S (2002) Insights into the G1/S in Plants. Planta **215**: 345-356

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An An Abidopsis thaliana T-DNA Mutagenized Population (GABI-Kat) for Flanking Sequence Tag-Based Reverse Genetics. Plant Mol Biol **53**: 247-259

Rotman N, Rozier F, Boavida L, Dumas C, Berger F, Faure JE (2003) Female Control of Male Gamete Delivery During Fertilization in *Arabidopsis thaliana*. Curr Biol **13**: 432-436

Sambrook J, Russell D (2001) Molecular Cloning: A Laboratory Manual. Ed 3rd. Cold Spring Harbor Laboratory Press, New York

Sarkar KR, Coe EH (1966) A Genetic Analysis of the Origin of Maternal Haploids in Maize. Genetics **54**: 453-464

Savidan Y (1980) Chromosomal and Embryological Analysis in Sexual x Apomictic Hybrids of *Panicum maximum* Jacq.Theor Appl Genet **57**: 153-156

Savidan Y (2000) Apomixis: Genetics and Breeding. Plant Breed Rev **18**:13-85

Savidan Y (2001) Transfer of Apomixis Through Wide Crosses. In Flowering of Apomixis: From Mechanisms to Genetic Engineering, Y Savidan, JG Carman, T DresselHaus, eds, Mexico: CIMMYT, IRD, European Commission DG VI, pp 153-167

Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A Leucine-Rich Repeat Containing Receptor-Like Kinase Marks Somatic Plant Cells Competent to Mark Embryos. Development **124**: 2049-2062

Schmid MW, Schmidt A, Klostermeier UC, Barann M, Rosenstiel P, Grossniklaus U (2012) A Powerful Method for Transcriptional Profiling of Specific Cell Types in Eucaryotes: Lasser-Assisted Microdissection and RNA Sequencing. PLoS ONE **7**: e29585

Schmidt A, Wuest SE, Vijverberg K, Baroux C, Kleen D Grossniklaus U (2011) Transcriptome Analysis of the Arabidopsis Megaspore Mother Cell Uncovers the Importance of RNA Helicases for Plant Germline Development. PLoS Biol **9**: e1001155

Schneiz K., Hulskamp M, Pruitt RE (1995) Wild-Type Ovule Development in *Arabidopsis thaliana*: a Light Microscope Study of Cleared Whole-Mount Tissue. Plant J **7**, 731-749

Schnitther A, Schöbinger U, Bouyer D, Weinl C, Stierhof YD, Hülskamp M (2002) Ectopic D-Type Cyclin Expression Induces Not Only DNA Replication but also Cell Division in Arabidopsis Trichomes. Proc Natl Acad Sci USA **99**: 6410-6415

Schwab R, Ossowski S, Reister M, Warthmann N, Weigel D (2006) Highly Specific Gene Silencing by Artificial MicroRNAs in *Arabidopsis.* Plant Cell **18**: 1121-1133

Schwab R, Ossowski S, Reister M, Warthmann N, Weigel D (2009) Directed Gene Silencing with Artificial MicroRNAs. In Mayers BC, Green PJ, eds, Plant MicroRNAs, Methods in Molecular Biology, pp 71-82

Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific Effects of MicroRNAs on the Plant Transcriptome. Dev Cell **8**: 517-527

Scott RJ, Spielman M, BaileyJ, Dickinson HG (1998) Parent-of-Origin Effects on Seed Development in *Arabidopsis thaliana.* Development **125**: 3329-3341

Scott RJ, Spielman M, Dickinson HG (2004) Stamen Structure and Function. The Plant Cell **16**, S46-S60

Sessions A, Burke E, Presting G, Aux G, McElver JJ, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C et al (2002) A High-Throughput Arabidopsis Reverse Genetic System Plant Cell **14**: 2985-2994

Shimotohno A, Ohno R, Bisova K, Sakaguchi N, Huang J, Koncz C, Uchimiya H, Umeda M (2006) Diverse Phosphoregulatory Mechanisms Controlling Cyclin-Dependent Kinase Activating Kinase in Arabidopsis. Plant J **47**: 701-710

Siddiqi I, Gamesh G, Grossniklaus U, Subbiah V (2000) The *Dyad* Gene is Required For Progression Through Female Meiosis in Arabidopsis. Development **127**: 197-207

Siles MM, Russell WK, Nelson LA, Baltensperger DD, Johnson B (2004) Heterosis for Grain Yield and Other Agronomic Traits in Foxtail Millet. Faculty Papers and Publications in Animal Sciences, University of Nebraska, Lincoln. Paper 156

Singh M, Goel S, Meeley RB, Dantec C, Parrinello H, Michaud C, Leblanc O, Grimanelli D (2011) Production of Viable Gametes without Meiosis in Maize Deficient for an ARGONAUTE protein. Plant Cell **23(2)**: 443-458

Sokolov VA, Independent Inheritance and Expression of Apomeiosis and Parthenogenesis in Maize-Gamma Grass Hybrids. Dokl Biol Sci **374**: 479-499

Spielman M, Vinkenoog R, Scott RJ (2003) Genetic Mechanisms of Apomixis. Phil Trans R Soc Lond B **358**: 1095-1103

Spillane C, Curtis MD, Grossniklaus U (2004) Apomixis Technology Development – Virgin Birth in Farmers Fields? Nature Biotechnology **22**: 687-691

Spillane C, Steimer A, Grossniklaus U (2001) Apomixis in Agriculture. Sex Plant Reprod **14**: 179-187

Stone SL, Kwong LW, Yee KM, Pelletier J, Leponiec L, Fischer RL, Goldberg RB, Harada JJ (2001) LEAFY COTYLEDON 2 Encodes a B3 Domain Transcription Factor that Induces Embryo Development. Proc Nat Acad Sci USA **98**: 11806-11811

Sun J, Niu QW, Tarkowski P, Zheng B, Tarkowska D, Sandberg G, Chua NH, Zuo J (2003) The Arabidopsis AtIPT8/PGA22 Gene Encodes an Isopentenyl Transferase that is Involved in de Novo Cytokinin Biosynthesis. Plant Physiol **131**: 167-176

Sundaresan V, Alandete-Saez M (2010) Pattern Formation in Miniature: The Female Gematophyte of Flowering Plants. Development **137(2)**: 179-189

Sundaresan V, Springer P, Volpe T, Haward S, Jones JD, Dean C, Ma H, Martienssen R (1995) Patterns of Gene Action in Plant Development Revealed by Enhancer Trap and Gene Trap Transposable Elements. Genes Dev **9**: 1797-1810

Suzuki G. (2009) Recent Progress in Plant Reproduction Research: The Story of the Male Gametophyte Through to Successful Fertilization. Plant Cell Physiol **50(11),** 1857-1864

Takahashi I, Kojima S, Norihiro S, Umeda-Hara C, Umeda M (2010) Two Arabidopsis Cyclins A3s Possess G1 Cyclin-Like Features. Plant Cell Rep **29**: 307-315

Tanni H, Chen X, Nurmberg P, Grant JJ, SantaMaria M, Chini A, Gilroy E, Birch PRJ, Loake GJ (2004) Activation Tagging in Plants: a Tool for Gene Discovery. Funct Interg Genomics **4**: 258-66

Tumbar T, Sudlow G, Belmint AS (1999) Large-Scale Chromatin Unfolding and Remodeling Induced by VP16 Acidic Activation Domain. J Cell Biol **145**: 1341-1354

Umeda M, Bhalearo RP, Schell J, Uchimiya H, Koncz C (1998) A Distinct Cyclin-Dependent Kinase-Activating Kinase of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **95**: 5021-5026

Umeda M, Shimotohno A, Yamaguchi M (2005) Control of Cell Division and Transcription by Cyclin-Dependent Kinase-Activating Kinases in Plants. Plant Cell Physiol **46**: 1437-1442

Uranga JA, Pedersen RA, Arechaga J (1996) Parthenogenetic Activation of Mouse Oocytes Using Calcium Ionophores and Protein Kinase C Stimulators. Int J Dev Biol **40**: 515-519

Vandepoele K, Raes J, De Veylder L, Rouze P, Rombauts S, Inze D (2002) Genome-Wide Analysis of Core Cell Cycle Genes in Arabidopsis. Plant Cell **14**: 903-916

van Dijk PJ, Bakx-Schatman JMT (2004) Formation of Unreduced Megaspores (Diplospory) in Apomictic Dandelions (*Taraxacum*) is Controlled by a Sex-Specific Dominant Gene. Genetics **166**: 483-492

van Dijk PJ, van Damme J (2000) Apomixis Technology and Paradox of Sex. Trends Plant Sci **5**:81-84

van Dijk PJ (2009) Apomixis: Basis For Non-Botanists. In Schön I, Martens K, van Dijk P, eds, Lost Sex. The Evolutionary Biology of Parthenogenesis. Springer, Dodrecht, pp 47-62

Verkest A, de O. Manes CL, Vercruysse S, Maes S, Van Der Schueren E, Beeckman T, Genschik P, Kuiper M, Inze D, De Veylder L (2005) The Cyclin-Dependent Kinase Inhibitor KRP2 Controls the Onset of the Endoreduplication Cycle During Arabidopsis Leaf Development Through Inhibition of Mitotic CDKA;1 Kinase Complex. Plant Cell **17**: 1723-1736

Vignalli M, Steger DJ, Neely KE, Workman JL (2000) Distribution of Acetylated Histones-Resulting from Gal4-VP16 Recruitment of SAGA and NuA4 Complexes. EMBO J 19: 2629-2640

Vijverberg K, Van der Hulst RGM, Lindhout P, van Dijk PJ (2004) A Genetic Linkage Map of the Diplosporous Chromosomal Region in *Taraxacum officinale* (Common Dandelion; Asteraceae). Theor Appl Genet **108**: 725-732

Vijverberg K, Milanovic-Ivanovic S, Bakx-Schotman van Dijk PJ (2010) Genetic Fine-Mapping of *Diplosporous* in *Taraxacum* (Dandelion; Asteraceae) Indicates a Duplicated DIP-gene. BMC Plant Biology **10**: 154

Vijverberg K, van Dijk PJ (2007) Genetic Linkage Mapping of Apomixis Loci. In E. Hörandl, U Grossniklaus, van Dijk PJ, Sharbel TF, eds, Apomixis: Evolution, Mechanisms and Perspectives. ARG Gantner Verlag, Rugell, Liechtenstein, pp 137-158

von Malek B, van der Graaff E, Scneitz K, Keller B (2002) The Arabidopsis Male Sterile Mutant dde2-2 is Deffective in the ALLENE OXIDE SYNTHASE Gene Encoding One of the Key Enzymes of the Jasmonic Acid Biosynthesis Pathway. Planta **216**: 187-192

Wang H, Qi Q, Schorr P, Cutler AJ, Crosby WL, Fowke LC (1998) ICK1, a Cyclin-Dependent Protein Kinase Inhibitor from *Arabidopsis thaliana* Interacts with both CdC2a and CycD3, and its Expression is Induced by Abscisic Acid. Plant J **15**: 501-510

Warthmann N, Chen H, Ossowski S, Weigel D, Herve P (2008) Highly Specific Gene Silencing by Artificial MicroRNAs in Rice. PLoS One **3**: e1829

Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrandiz C, Kardailsky I, Malanchruvil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation Tagging in Arabidopsis. Plant Physiol **122**: 1003-1-1013

Weterings K, Russell SD (2004) Experimental Analysis of the Fertilization Process. The Plant Cell, **16**. S107-S118

Wissman E, Hartmann U, Sagasser M, Baumann E, Oalme K, Hahlbrock K, Saedler H, Wiesshaar B (1998) Knock-Out Mutants From an En-1 Mutagenized Arabidopsis thaliana Population Generate Phenylpropanoid BBiosynthesis Phenotypes. Proc Natl Acad Sci USA **95**: 12432-12437

Wolfe KW, Liu Q (1999) The Maize Mutant Polymitotic Arrest Cell Cycle Events During Microspore Development. Planta **210**: 27-33

Wuest SE, Vijverberg K, Schmidt A, Weiss M, Gheyselinck J, Lohr M, Wellmer F, Rahnenführer J, von Mering C, Grossnikluas U (2010) *Arabidopsis* female

Gametophyte Gene Expression Map Reveals Similarities Between Plant and Animal Gametes. Curr Biol **20**: 506-512

Xu YY, Wang XM, Li J, Li JH, Wu JS, Walker JC, Xu ZH, Chong H (2005) Activation of the WUS Gene Induces Ectopic Initiation of Floral Meristems on Mature Stem Surface in *Arabidopsis thaliana*. Plant Mol Biol **57**: 773-784

Yadegari R, Drews GN (2004) Female Gametophyte Development. Plant Cell **16**: S133-141

Yadegari R, Paiva G, Laux T, Koltunow AM, Apuya N, Zimmerman JL, Fischer RL, Harada JJ, Goldberg RB (1994) Cell Differentiation and Morphogenesis Are Uncoupled in Arabidopsis Raspberry Embryos. Plant Cell **6**: 1713-1729

Yamada-Akiyama H, Akiyama Y, Ebina M, Xu Q, Tsuruta S, Yazaki J, Kisimoto N, Kikuchi S, Takahara M, Takamizo T, Suqita S, Nakagawa H (2009) Analysis of Expressed Sequence Tags in Apomictic Guinea Grass (*Panicum maximum*). J Plant Physiol **166(7)**: 750-761

Yamada-Akijama H, Akiyama Y, Ebina M et al (2009) Analysis of Expressed Sequence Tags In Apomictic Guineagrass (*Panicum maximum*). J Plant Physiol **166**: 750-761

Yang W, Jefferson RA, Huttner E, Moore JM, Gagliano WB, Grossniklaus U (2005) An Egg Apparatus-Specific Enhancer of Arabidpsis, Identified by Enhancer Detection. Plant Physiol **139**: 1421-1432

Ye GN, Stone D, Pang SZ, Creely W, Gonzalez K, Hinchee M (1999) Arabidopsis Ovule is the Target for Agrobacterium in Planta Vacuum Infiltration Transformation. Plant J **19**:249-257

Zhou Y, Wang H, Gilmer S, Whitwill S, Fowke LC (2003) Effects of Co-Expressing the Plant CDK Inhibitor ICK1 and D-Type Cyclin Genes on Plant Growth, Cell Size and Ploidy in *Arabidopsis thaliana*. Planta **216**: 604-13

Zuo J, Niu QW, Chua NH (2000) Technical Advance: An Estrogen Receptor-Based Transactivator XVE Mediates Highly Inducible Gene Expression in Transgenic Plants. Plant J **24**:265-273

Zuo J, Niu QW, Frugis G, Chua NH (2002) The *WUSCHEL* Gene Promotes Vegetative-to-Embryonic Transition in Arabidopsis. Plant J **30**: 349-359